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1988 TOXIC HAZARDS RESEARCH UNIT ANNUAL REPORT

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HARRY G. ARMSTRONG AEROSPACE MEDICAL RESEARCH LABORATORY
HUMAN SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433-6573

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This summary represents the statements and opinions of the participants and does not necessarily reflect the policy or position of the Department of Defense and the separate services, the Suppliers of Advanced Composite Materials Association (SACMA), the Aerospace Industries Association (AIA), or the member companies of these organizations.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

MICHAEL B. BALLINGER, Lt Col, USAF, BSC
Chief, Toxic Hazards Division
Harry G. Armstrong Aerospace Medical Research Laboratory

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06	11		

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This report has been prepared as a review of the activities of the Toxic Hazards Research Unit for the period of October 1987 through September 1988. Research activities focused on the toxicity evaluations of aerospace chemicals and materials, studies on Installation Restoration Program chemicals, studies on Air Force Fuels, development of physiologically based pharmacokinetic and pharmacodynamic models, studies of chemical defense materials and associated training simulants, subchronic studies on materials of interest to the Navy, and assessment of the delayed neurotoxicity potential of shipboard hydraulic fluid samples. The review of the studies presented here includes partially completed efforts that will be reported further in subsequent annual reports.

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Repeated Inhalation Toxicity
Repeated Oral Toxicity
Sensitization and Acute Dermal Irritation
Sister Chromatid Exchanges

PREFACE

The 25th Annual Report of the Toxic Hazards Research Unit (THRU) presents research and support efforts conducted by NSI Environmental Sciences (NSI-ES) on behalf of the U.S. Air Force and the U.S. Navy under Contract Number F33615-85-C-0532. It should be noted that during the course of the contract year, Northrop Corporation divested itself of Northrop Services Incorporated, including Environmental Sciences, which was acquired by ManTech International Corporation. The name under ManTech International was changed to NSI Technology Services Corporation – Environmental Sciences. This name change has had no impact on the term of the existing contract or on the conduct of studies in the THRU. This document represents the third report for the current THRU contract and describes accomplishments from October 1987 through September 1988.

Operation of the THRU under this contract was initiated in January 1986 under Project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Task 00, Toxic Hazards Research, Work Unit number 63020001. This research effort is co-sponsored by the Naval Medical Research and Development Command under the Naval Medical Research Institute, Toxicology Detachment, Navy Work Unit M0096-004-0006, "Criteria for Exposure Limits in the Navy Operational Environment." The objectives of the research program are to identify and characterize the toxic effects of chemicals and materials that are of operational interest and concern to the Air Force and Navy. This coordinated dual-service program generates scientific information from which computer simulation models of toxicity can be developed to drive experimental design and to assist in risk assessments.

Melvin E. Andersen, Ph D., Acting Director of the Toxic Hazards Division/Senior Staff Scientist, Toxic Hazards Division, Harry G Armstrong Aerospace Medical Research Laboratory, was the Contract Technical Monitor during the period of this report.

Commander David A Macys, MSC, USN, was the director of the U S Navy portion of this contract during the period of this report.

Raymond S. Kutzman, Ph.D., assumed Program Manager responsibilities and the Directorship of the THRU in April 1988 for NSI-ES Prior to that time, William E.Houston, Ph.D., directed the THRU The preparation of this report represents major contributions by the NSI-ES staff of the THRU.

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SECTION 1

LIST OF ABBREVIATIONS

AALAS	-	American Association for Laboratory Animal Science
AAMRL	-	Armstrong Aerospace Medical Research Laboratory
AChE	-	Acetylcholinesterase
ACSL	-	Advanced Continuous Simulation Language
AGT	-	Average generation time
AO	-	Acridine orange
Be	-	Beryllium
BeO	-	Beryllium oxide
BrdUrd	-	Bromodeoxyuridine
BSA	-	Bovine serum albumin
BuChE	-	Butyrylcholinesterase
BUN	-	Blood urea nitrogen
CaP	-	Calcium-phosphorus
CaE	-	Carboxylesterase
CHLF	-	Chloroform
CDNB	-	Chlorodinitrobenzene
CPFB	-	Chloropentafluorobenzene
CTFE	-	Chlorotrifluoroethylene
CTP	-	Cyclotriphosphazene
DCE	-	Dichloroethane
DFP	-	Diisopropylfluorophosphate
DMBA	-	7,12-dimethylbenz(a)anthracene
DMEM	-	Dulbecco's Modified Eagles Medium
DNA	-	Deoxyribonucleic acid
ECD	-	Electron capture detector

F-344	-	Fischer 344 rats
FBS	-	Fetal bovine serum
g	-	Gravity
GC	-	Gas chromatography
GC/MS	-	Gas chromatography/mass spectrometry
GLP	-	Good laboratory practices
HBSS	-	Hank's balanced salt solution
HBSSA	-	A modified Hank's balanced salt solution for washing primary hepatocytes
HC 27-S	-	Halocarbon 27-S
ip	-	Intraperitoneal
IRP	-	Installation Restoration Program
iv	-	Intraveneous
IVPL	-	Isolated ventilated perfused lung
JMEM		Joklik's Modified Minimum Essential Medium
LDH	-	Lactate dehydrogenase
M-CHL	-	Methylene chloride
MFV	-	Minimum fluidizing velocity
MI	-	Mitotic index
MN	-	Micronuclei
MSDS	-	Manufacturer's safety data sheet
NCE	-	Normochromatic erythrocytes
NIOSH	-	National Institute for Occupational Safety and Health
NMRI/TD	-	Naval Medical Research Institute/Toxicology Detachment
NSI-ES	-	NSI Technology Services Corporation – Environmental Sciences
OP	-	Organophosphate
OPIDN	-	Organophosphorus-induced delayed neuropathy
OPM	-	Pharmacodynamic model
PB-PK	-	Physiologically based pharmacokinetic

PBS	-	Phosphate-buffered saline
PCE	-	Polychromatic erythrocytes
PFDA	-	Perfluorodecanoic acid
QA	-	Quality Assurance
RBC	-	Red blood cell
RER	-	Rough endoplasmic reticulum
RI	-	Replicative index
RTE	-	Rat tracheal epithelial
RTP	-	Research Triangle Park, NC
SCE	-	Sister chromatid exchange
SEM	-	Standard error of the mean
SER	-	Smooth endoplasmic reticulum
SGOT	-	Serum glutamic oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic transaminase
TCA	-	Trichloroacetic acid
TCD	-	Thermal conductivity detector
TCE	-	Trichloroethylene
TCOH	-	Trichloroethanol
TEM	-	Transmission electron microscopy
THRU	-	Toxic Hazards Research Unit
TM-1-P OH	-	2,3,4-Trimethyl-1-pentanol
TM-2-P OH	-	2,3 4-Trimethyl-2-pentanol
TMP	-	2,3,4-Trimethylpentane
TMPA	-	2,3,4-Trimethyl-1-pentanoic acid
TOCP	-	Triorthocresyl phosphate

SECTION 2

INTRODUCTION

Research activities and support efforts of the THRU are conducted as a continuing program independent of contract years. Technical directives are established by the U.S. Air Force and U.S. Navy customers and define areas of investigation and support to be provided under the contract The contents of this report are organized according to the technical directive areas of investigation.

Research efforts on the toxicity of aerospace chemicals and materials has been dominated by work on chlorotrifluoroethylene (CTFE) oligomer This nonflammable, saturated, hydrogen-free chlorofluorocarbon oil is being considered for use as a hydraulic fluid in advanced Department of Defense hardware. Initial acute studies of CTFE indicated low toxicity. The results of subsequent studies are reported here. In addition to investigations on this hydraulic fluid, studies were also conducted on the skin irritation and sensitization potential of candidate fabrics for the production of clothing that would provide personnel protection in high temperature or fire situations.

There is continued interest in the hazard posed by chemicals frequently identified at Installation Restoration Program (IRP) sites. These chemicals are often volatile and enter the body via inhalation. Therefore, the metabolizing ability of the lung for these chemicals needs to be assessed to develop simulation models that can predict the amount of chemical reaching potential target organs. An isolated ventilated perfused lung apparatus was prepared and ventilated with an atmosphere containing trichloroethylene The presence of metabolites was assessed in the perfusate In the case of this frequently identified IRP chemical, the lung demonstrated very limited metabolic capacity.

Two candidate fuels were studied under the directive to assess the toxicity of such materials. Trimethylpentane has been used as a model compound for examining the induction of hydrocarbon-induced nephropathy in male rats Studies were conducted to assess the ability of both liver and kidney cells to metabolize trimethylpentane in efforts to better understand the role of these two organs in the pathogenesis of the renal lesion An Air Force interest in beryllium as a rocket fuel resulted in studies to compare the relative toxicity of the exhaust particles from beryllium-fueled rockets with that of commercially available beryllium oxides. These studies examined the ability of the beryllium materials to induce transformation and DNA breaks in the respiratory epithelial cells of rats and their relative solubilities in artificial pulmonary fluid

Pharmacokinetic and pharmacodynamic modeling efforts reported here include work on the gastrointestinal absorption of chloroform This chemical is a commonly identified environmental

contaminant as well as a carcinogen A chloroform carcinogenicity model is being developed to assess the human risk associated with this compound.

The Air Force is concerned with the impact of low concentration exposure to CW agents on the capabilities of personnel. A computer simulation model of the toxic effects of the organophosphate diisopropylfluorophosphate, an acetylcholinesterase inhibitor, was developed for use in assessing the risk from exposure to agents with similar mechanisms of toxic action. Data sets from the literature were used for model development and validation.

Chloropentafluorobenzene (CPFB) has dominated the efforts to identify a chemical that can be used to simulate a CW agent for training purposes. To be used for this purpose the chemical must be readily detectable in air, blood, and urine and have little or no toxicity potential. Acute toxicity studies with CPFB have demonstrated that CPFB has limited adverse effects in laboratory rodents. The studies reported here include a 21-day repeated inhalation exposure to assess the systemic toxicity as well as the *in vivo* genotoxic potential of CPFB.

Several repeated treatment studies were conducted for the U S Navy on hydraulic and lubricating fluids. Among these were repeated inhalation and dermal exposures to cyclotriphosphazene, a candidate hydraulic fluid. This material did not demonstrate acute toxicity potential in earlier, oral tests, skin and eye irritation studies, or skin sensitization assays. Therefore, more extensive investigations were initiated to determine the results following repeated treatment with this fluid. The toxicity of Halocarbon 27-S (HC 27-S) was assessed using a repeated dosing regimen. This lubricating oil consists of CTFE oligomers with a longer average chain length than CTFE. The structural similarity between CTFE and HC 27-S, however, suggests that similar chronic effects might be expected

Following the development of neurological symptoms by a Navy crewman, samples of shipboard hydraulic fluids were assessed for their potential to induce triorthocresyl phosphate-like symptoms in hens. This study showed that the shipboard hydraulic fluids did not cause the type of toxic effects seen in the crewman

In addition to the conduct of scientific investigations, the THRU sponsors an annual meeting on toxicology The title of the 18th Conference on Toxicology, held during this report period, was "Contemporary Initiatives in Quantitative Toxicology " The proceedings of this conference are published under separate cover and will not be discussed in this report

Other contract activities not specific to research efforts are covered in the appendices of this report Appendi notation is not designed to diminish the importance of these efforts They are, in

fact, absolute administrative and support requirements for the conduct of the studies appearing in the body of this report.

SECTION 3

TOXICOLOGY EVALUATIONS OF AEROSPACE CHEMICALS AND MATERIALS

3.1 ACUTE SKIN IRRITATION AND SENSITIZATION POTENTIAL OF CHLOROTRIFLUOROETHYLENE

E. R. Kinkead and B.T. Culpepper

INTRODUCTION

Chlorotrifluoroethylene (CTFE) oligomer is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil It is noncorrosive, has high thermal stability, good lubricity, and high dielectic strength Recent dermal and inhalation studies indicated that CTFE has a low degree of toxicity. There were no deaths among rabbits dermally exposed to 2 g CTFE/kg body weight (Gargus, 1983), and there were no deaths among rats exposed for 4 h to saturated-vapor concentrations of CTFE (Coate, 1984; Kinkead et al , 1987)

Following oral and inhalation exposures, CTFE was readily absorbed and fluoride was subsequently excreted in urine Plasma and urine fluoride levels remained elevated for more than one week following oral exposure and for at least 24 h following inhalation exposure However, CTFE absorption was not evident following dermal exposure (Kinkead et al., 1987) Histopathologic examination of nerve tissue from hens dosed with CTFE showed no lesions of the type seen in organophosphate toxicity The acute toxicity evaluation of CTFE compares favorably with that of other hydraulic fluids tested in this laboratory However, the irritation and sensitization potential or effects associated with repeated or continuous exposure to CTFE have not been investigated.

In this study data were collected to provide information on the irritation and sensitization potential of CTFE.

METHODS AND EXPERIMENTAL EVALUATIONS

A detailed description of the methods and experimental evaluations performed for this study was provided in the 1987 Toxic Hazards Research Unit Annual Report (Kinkead et al , 1988)

Eye Irritation Assessment

Nine female New Zealand White rabbits, weighing 2 to 3 kg, were examined with fluorescein stain prior to use to ensure the absence of lesions or injury A topical anesthetic (Opthetic, Allergan Pharmaceuticals, Inc , Irvine, CA; Proparacaine HCl 0 5%) was instilled in the eyes, treated and control, of all rabbits approximately 2 min prior to application of the test material One-tenth of a milliliter of the test material was applied to one eye of each of the nine albino rabbits. The opposite eye was left untreated and served as the control The treated eye of three rabbits was flushed with

lukewarm de-ionized water for 1 min starting 30 sec after instillation. The treated eyes of the remaining six rabbits were not flushed Examinations for gross signs of eye irritation were made at 1, 24, 48, and 72 h following treatment. Irritation was scored according to the method of Draize (1944) (Appendix 1), in which the total score for the eye is the sum of the cornea, iris, and conjunctiva scores

Skin Irritation Assessment

Six female New Zealand White rabbits were clipped on the back and sides 24 h prior to treatment to allow for recovery of the skin from any abrasion resulting from the clipping The test agent was applied in the amount of 0 5 mL to a designated patch area and was covered by a 2 5-cm square of surgical gauze two single layers thick. The gauze patch was held in place with strips of elastoplast tape, and the entire area was covered with dental dam and secured with Vetrap (3M Corp., Minneapolis, MN) and elastoplast tape. The patches remained in place for 4 h, at which time all wrappings were removed and the residual test agent was wiped from the animals. The test areas were evaluated for irritation using the Draize Scoring System (1944) (Appendix 2) as a reference standard at 4, 24, 48, and 72 h. The total score of the four observations for all rabbits was divided by 24 to yield a primary irritation rating that was interpreted using the National Institute for Occupational Safety and Health skin test rating (Appendix 3).

Sensitization Assessment

Ten male guinea pigs were dosed with 0 1 mL of the test material on the clipped left flank. The site of the sensitization test was an area just behind the shoulder girdle The site was clipped with an Oster® animal clipper and depilated with a commercial depilatory (Surgex Hair Remover Cream, Sparta Instrument Corp , Hayward, CA) 4 h prior to treatment. A Vetrap frame with a 1.5 x 1.5-cm opening at the site of the depilated area was affixed to the guinea pig. The test material was topically applied to the test area, covered with gauze, dental dam, and adhesive tape. This was done on Mondays, Wednesdays, and Fridays until a total of four sensitizing treatments were applied. In addition to the third sensitizing treatment, 0 2 mL of a 50% aqueous dilution of Freund's adjuvant (Sacto Adjuvant Complete, Freund, Difco Laboratories, Detroit, MI) per animal was injected intradermally using two or three sites next to the test site on each animal. Following the fourth sensitizing dose, the animals were rested for two weeks Both flanks were clipped again and each animal was challenged on one flank with the test material. The challenge application was not occluded The skin response at these sites was recorded at 24 and 48 h after application (method in Appendix 4) Any animal eliciting a score of two or more at the test solution challenge site for the 48-h scoring was rated a positive responder The frequency of the reaction is the important statistic in determining sensitization potential (Appendix 5)

RESULTS

Eye irritation

Nine animals were used in this study In prescreening the rabbit eyes with fluorescein stain prior to treatment, several showed mild to intense staining of the corneal epithelium. The eyes showing opacity were not used in the study.

Mild conjunctival redness was present in the eyes of six rabbits 1 h after treatment Redness was present in one rabbit at the 48-h evaluation, but no rabbits showed conjunctival redness at the 24- or 72-h evaluation. One rabbit exhibited mild chemosis and another exhibited mild discharge 1 h after treatment However, neither chemosis nor discharge was present at the 24-, 48-, or 72-h evaluations

Skin irritation

Following 4 h of skin contact with the test compound, the rabbits were unwrapped and the residual test agent was wiped off. At 4-h postexposure no animal exhibited any sign of erythema, edema, or necrosis. All animals received scores of 0 for the remaining 24-, 48-, and 72-h evaluation periods classifying this test compound to be nonirritating

Sensitization

Ten guinea pigs were treated with the test agent during the challenge applications. Six animals exhibited very slight erythema (score of 1), and one exhibited slight erythema (score of 2) at the initial challenge (Table 3.1-1). A score of 2 or above denotes a positive responder. In questionable sensitized guinea pigs, rechallenge is an effective confirmatory step in determining sensitization (Gad et al., 1986), therefore a rechallenge was performed two weeks following the initial challenge application. The 48-h rechallenge score revealed three animals to be positive responders Based on the scale for determining sensitization potential (Appendix 5), the 30% (three of 10 animals) sensitization rate would classify CTFE as a mild sensitizing agent

DISCUSSION

The application of CTFE to intact rabbit skin produced no signs of irritation, however, continued skin contact could produce an allergic response in sensitive individuals. The fluid produced mild conjunctival redness in rabbit eyes 1 h following application, but this was resolved by 24 h. It can be assumed that the fluid would not be irritating to human skin but would be irritating upon accidental eye contact Washing the eyes immediately after contact does not preclude the transient irritating effects.

TABLE 3.1-1. RESPONSE OF GUINEA PIGS TO CHALLENGE DOSE OF CTFE

Guinea Pig No.	Initial Challenge 48 h Reaction Score	Rechallenge 48 h Reaction Score
170	2	2
171	1[a]	1
173	1	0
175	1	1
176	1	1
177	0	0
178	1	2
179	0	0
182	0	1
183	1	2

[a] A score of less than 2 is not rated a positive responder

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APPENDIX 1

DRAIZE[a] SCALE FOR SCALING OCULAR LESIONS

Parameter	Score
1. Cornea	
A Opacity degree of density (area most taken for reading)	
No opacity	0
Scattered or diffuse area, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obsured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisibile	4
B. Area of cornea involved	
One-quarter (or less), but not zero	1
Greater than one-quarter, but less than one-half	2
Greater than one-half, but less than three-quarters	3
Greater than three-quarters, up to whole area	4
Score = A x B x 5 Total Maximum =	80
2. Iris	
A Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or a combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
Score = A x 5 Total Maximum =	10
3. Conjuctivae	
A Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
No swelling	0
Any swelling above normal (included nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
Score = (A + B + C) x 2 Total Maximum =	20
The TOTAL MAXIMUM SCORE is the sum of all scores obtained for the cornea, iris, and conjunctivae	
Maximum Total Score Possible =	110

[a] Draize, J H , G Woodward, and H O Calvery 1944 Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes *J Pharm Exp Therap* 32 377 390

APPENDIX 2

DRAIZE[a] SCALE FOR EVALUATING AND SCORING SKIN REACTIONS

Parameter	Score
1. Erythema	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness)	4
2. Edema	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raising approx. 1 mm)	3
Severe edema (raising more than 1 mm and extending beyond area of exposure)	4
3. Necrosis[b]	
No necrosis	0
Slight necrosis (less than one-fourth of the exposed area)	5
Moderate necrosis (one-fourth to one-half of the exposed area)	10
Severe necrosis (more than one-half of the exposed area)	15

[a] Draize et al., 1944

[b] Necrosis, for the purpose of this scoring system, is defined as a chemical denaturation of tissue sufficiently severe to result in fibrotic replacement (scar tissue). Superficial eschar, which heals without scarring, is not classified as necrosis.

APPENDIX 3

NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH INTERPRETATION OF SKIN TEST RATINGS[a]

	Rating	Interpretation
Intact Skin	0 – 0.9	Nonirritant; probably safe for human skin contact
	1 – 1 9	Mild irritant; may be safe for use, but appropriate protective measures are recommended during contact
	2 – 4	Too irritating for human skin contact; avoid contact

[a] Campbell, K I , E L George, L L Hale, and J F Stara 1975 Dermal Irritancy of Metal Compounds *Arch. Environ Health* 30· 168-170

APPENDIX 4

GRADING SYSTEM[a] FOR SENSITIZATION TEST

Erythema	Score	Edema	Score
None	0	None	0
Very Slight Pink	1	Very Slight	1
Slight Pink	2	Slight	2
Moderate Red	3	Moderate	3
Very Red	4	Marked	4

[a] Toxic Hazards Research Unit grading system for sensitization test

APPENDIX 5

SCALE[a] FOR DETERMINING SENSITIZATION POTENTIAL

Sensitization Rate (%)	Grade
10	Weak
20-30	Mild
40-60	Moderate
70-80	Strong
90-100	Extreme

[a] Toxic Hazards Research Unit scale for determining sensitization potential

3.2 INHALATION EXPOSURE TO CHLOROTRIFLUOROETHYLENE OLIGOMER-EXPOSURE ATMOSPHERE GENERATION AND CHARACTERIZATION

R.L. Carpenter, E.C. Kimmel, and C.R. Doarn

ABSTRACT

Chlorotrifluoroethylene (CTFE) hydraulic fluid was tested for inhalation toxicity in a 90-day subchronic study. The exposure atmosphere was generated using nebulization methods and resultant atmospheres were characterized for both vapor and aerosol composition. CTFE was found to divide into two distinct sets of peaks by gas chromatography (GC). The aerosol phase of the chamber atmosphere was enriched in material contributing to the second of these sets of peaks. Exposure chamber concentrations were maintained to within 4% of target value during the study

INTRODUCTION

The CTFE 3.1 oil used in this study is one of a class of totally halogenated oils having properties useful as lubricants and hydraulic fluids. They are produced by polymerizing CTFE, stopping the polymerization reaction with the addition of chlorine to the ends of the growing molecular chains, and separating the reaction mixture into fractions The resulting materials are selected to meet specific engineering criteria and mixed with additives to result in usable fluids.

This report is one of a series that describes a 90-day inhalation toxicity evaluation of one such oil, a hydraulic fluid intended for military applications. The inhalation chamber atmosphere present during the exposure phase of the toxicology study is described. The development of analytical methods to quantify the CTFE in blood, urine, and tissue, as well as in the exposure chamber atmosphere, is described elsewhere, as are the toxicological results.

CTFE is a semi-volatile material having sufficiently high vapor pressure that both the vapor and aerosol phases of the exposure atmosphere required quantitation in order to define the inhaled dose to the animals in the study. Rats were exposed to 0 25, 0 50, and 1.00 mg/L of total CTFE vapor and aerosol. Exposures were carried out for 6 h per day, five days per week for 90 calendar days

MATERIALS AND METHODS

CTFE inhalation exposure atmospheres were generated using a Collison nebulizer to produce a mixture of aerosol and vapor phases that was introduced into each chamber through a mixing section in the chamber air supply. The nebulizer operating parameters were chosen to maintain the ratio of aerosol to vapor as nearly equal among the chambers as possible while consistent with maintaining the desired target total CTFE concentrations This system is illustrated in Figure 3.2-1.

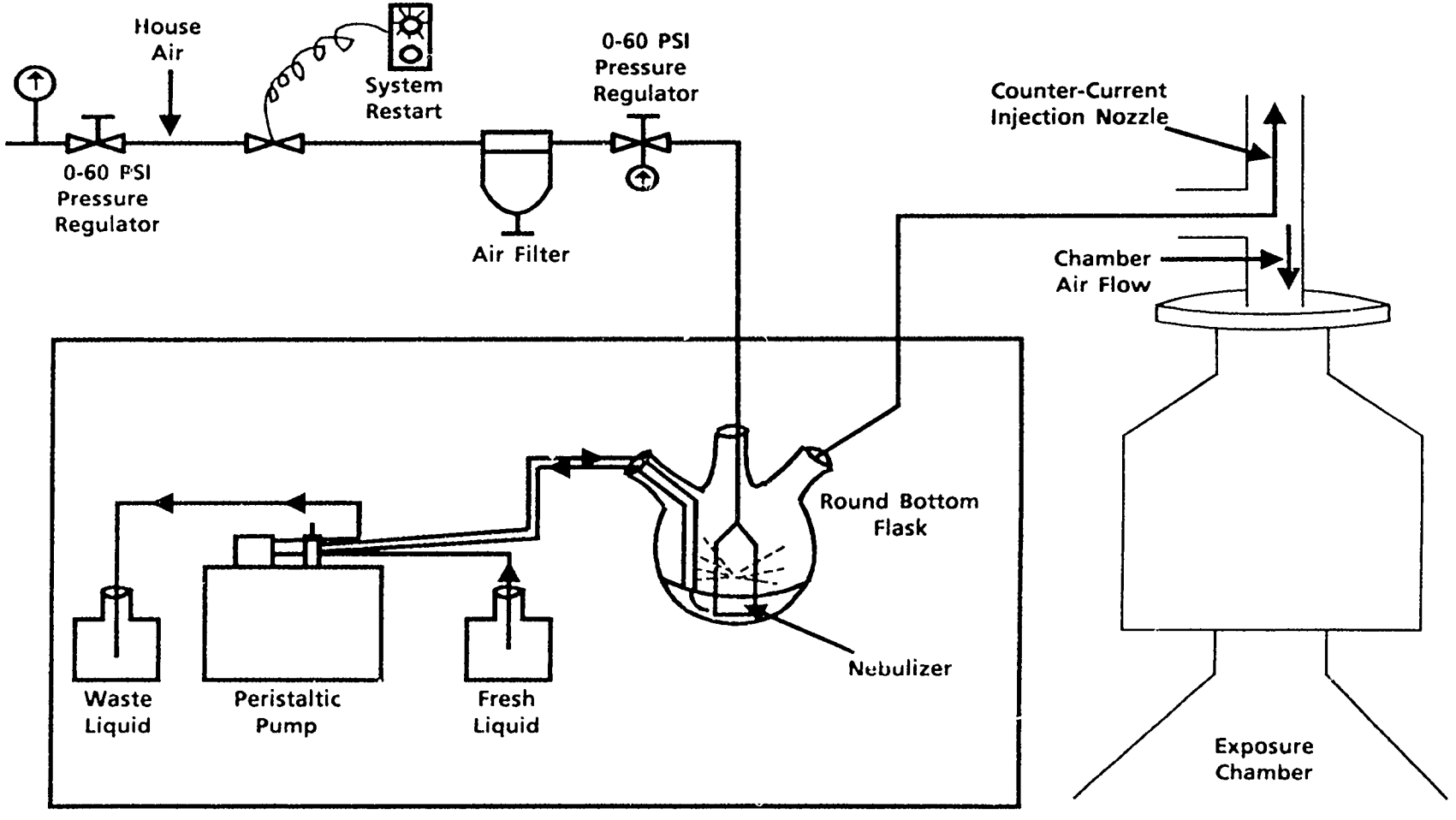

Figure 3.2-1. Typical Generation System – Chlorotrifluoroethylene.

Exposure chamber CTFE contents were quantified each day during the exposure phase of the study. Figure 3.2-2 describes the chamber sampling system used to obtain the needed samples

The chamber aerosol characteristics were determined using a cascade impactor designed at the THRU following the criteria for reliable operation established by Marple (1974) Collected material was quantified gravimetrically, and the weights obtained were analyzed by the method of weighted-least squares to obtain mean aerosol particle aerodynamic diameter and the distribution geometric standard deviation (Hill, 1977). Aerosol concentration was determined by collecting and weighing the aerosol contained in a known volume of air.

GC was used to quantify the mixture of CTFE oligomers as described elsewhere in this report. The aerosol material collected by impaction was analyzed to produce data describing the composition of each aerosol size fraction. Vapor samples were analyzed also for oligomer content by GC. Grab samples were collected in a gas-tight syringe containing hexane as a solvent. The collected vapor was diluted further in hexane and injected into a GC.

RESULTS

Overall CTFE concentrations and aerosol properties in the three exposure chambers are summarized in Table 3.2-1. Figures 3 2-3 through 3 2-8 illustrate the daily variation of the exposure and aerosol properties. Figure 3.2-9 shows the ratio of overall aerosol to vapor concentration for each chamber.

GC analysis revealed that CTFE separates into two distinct sets of peaks. Figure 3.2-10 is a chromatograph of CTFE standard Chromatography of a chamber atmosphere aerosol fraction revealed that the aerosol material is depleted in material corresponding to the first set of peaks (Figure 3.2-11).

Within the aerosol phase, GC illustrated that particle CTFE composition was relatively constant. Figure 3 2-12 shows the percent of total GC peak area represented by the eight significant peaks present in the late eluting group. For comparison, the same data are shown for the CTFE standard and the filter sample collected at the same time

DISCUSSION

The integrated CTFE generation and analysis system operated successfully for 90 days, producing a closely regulated exposure of stable composition The data obtained indicate that CTFE aerosolization produces a mixed atmosphere containing CTFE vapor enriched in the material that elutes early in a GC The aerosol phase of this atmosphere is enriched in material which elutes late in a GC The ratio of aerosol to vapor material for the exposure varied over a factor of two for a four-fold change in concentration (Figure 3 2-9) Thus, the fraction of material present as an aerosol is concentration driven These observations imply that atomization of small amounts of CTFE into the atmosphere will produce vapor exposures upon inhalation, while atomization of large amounts of

CTFE will result in mixed aerosol/vapor exposures In these two situations, the inhaled CTFE dose will differ both in quantity and composition.

REFERENCES

Hill, M.S., C.R. Watson, and O.R. Moss. 1977 Newcas - An Interactive Computer Program for Particle Size Analysis; Battelle Pacific Northwest Laboratories , PHL-2405

Marple, V.A., B.Y.H. Liu, and K.T. Whitby. 1974 Fluid mechanics of the laminar flow aerosol impactor. *Aerosol Science,* pp. 1-16.

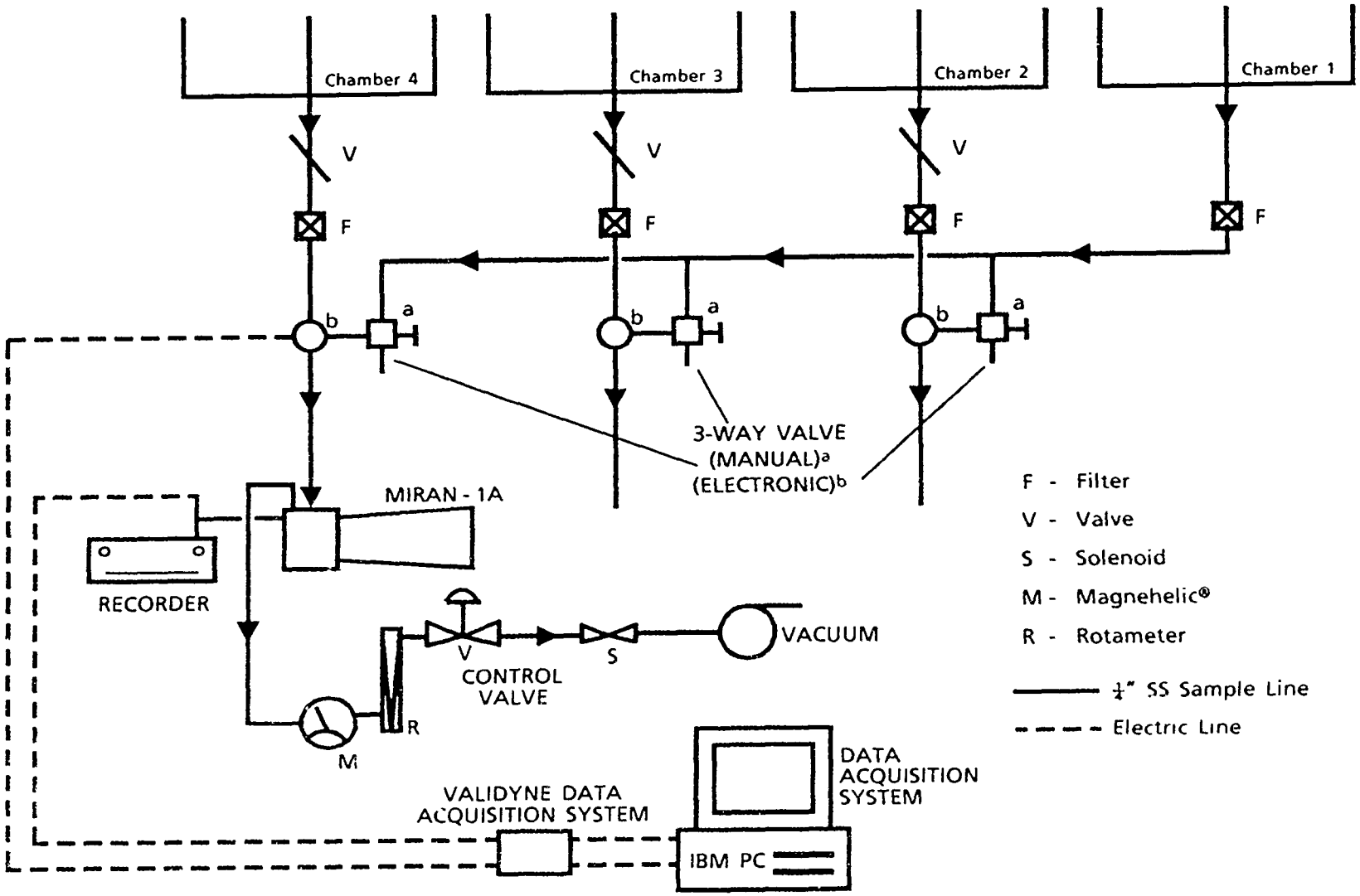

Figure 3.2-2. Sampling System – Chlorotrifluoroethylene.

TABLE 3.2-1. CTFE EXPOSURE CHAMBER CONCENTRATIONS DURING 90-DAY INHALATION EXPOSURE

Target Concentration (mg/L)	0 25	0.50	1.00
Mean Concentration (mg/L)	0 25	0.48	0.98
Standard Error	0 002	0 003	0.004
Lowest Daily Average (mg/L)	0.21	0.43	0.93
Highest Daily Average (mg/L)	0.28	0.53	1.14
Percent Aerosol	5 3	6 8	8.9
Aerosol MMAD (micrometers)	1 2	0 97	1 14
Aerosol Geom. Std. Dev	2 6	2.2	2.2

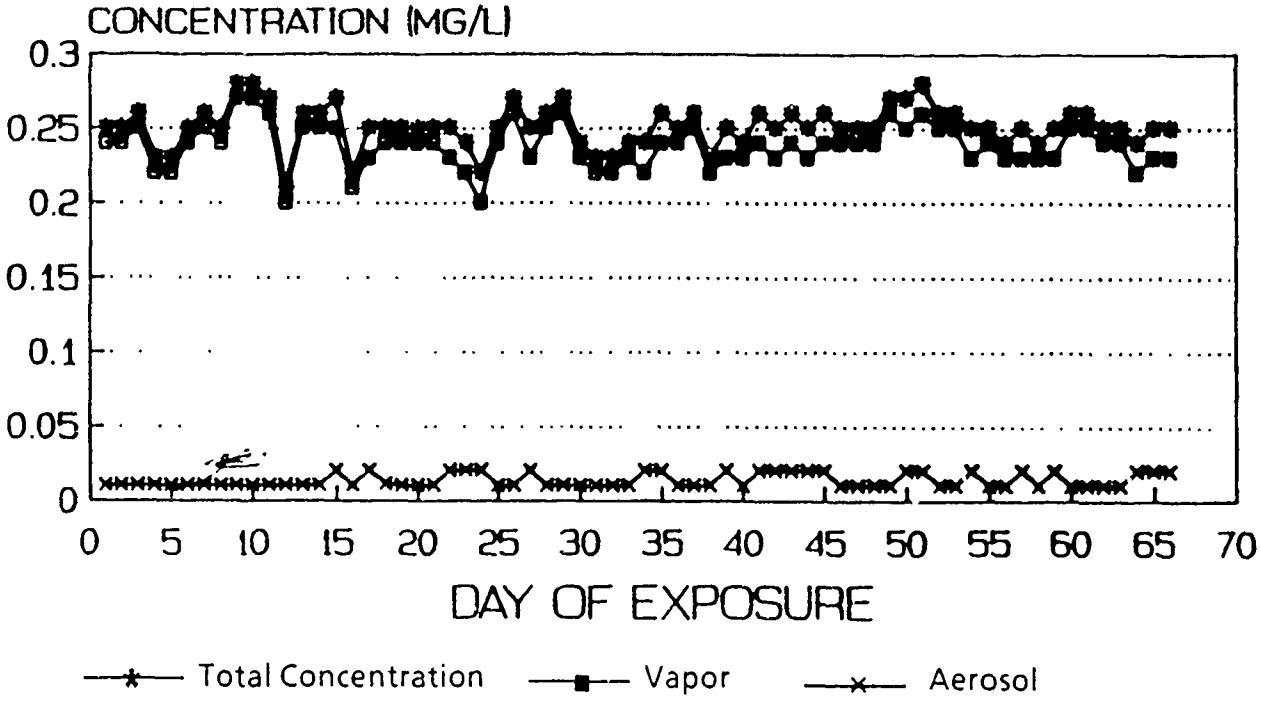

Figure 3.2-3. Concentration vs. Day of Exposure – Chlorotrifluoroethylene (0.25 mg/L).

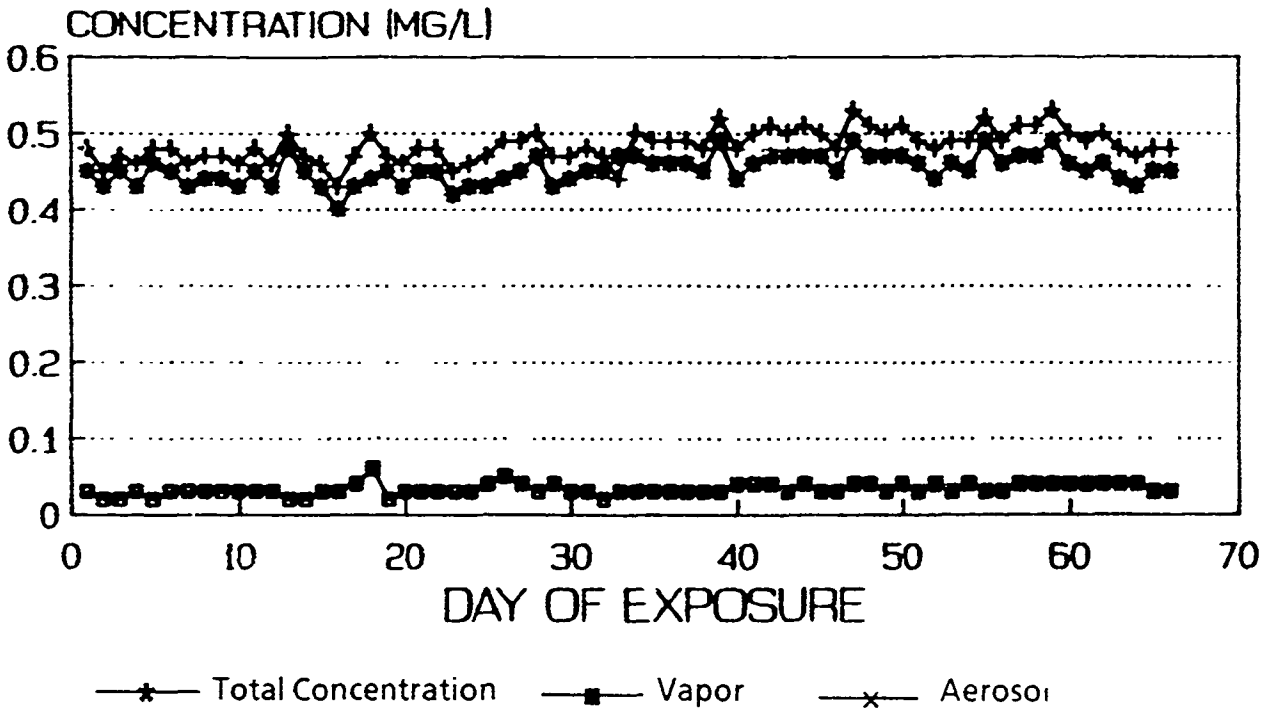

Figure 3.2-4. Concentration vs. Day of Exposure – Chlorotrifluoroethylene (0.50 mg/L).

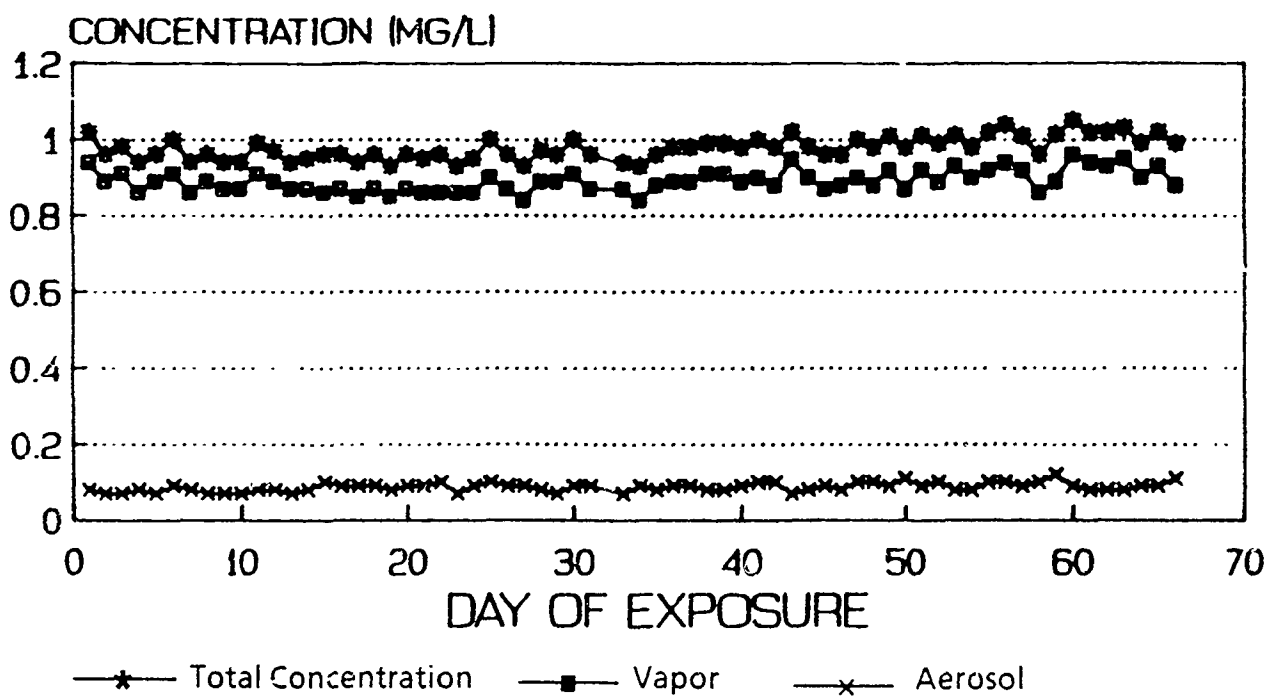

Figure 3.2-5. Concentration vs. Day of Exposure – Chlorotrifluoroethylene (1.00 mg/L).

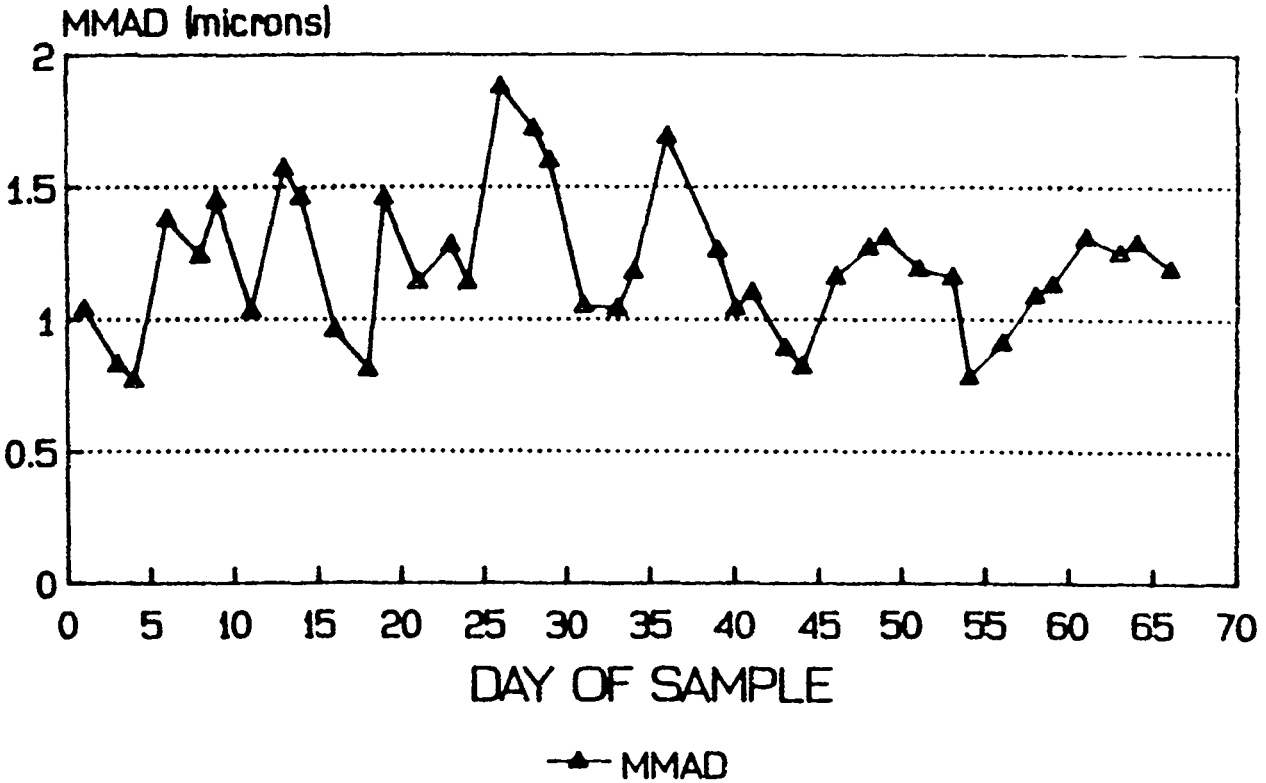

Figure 3.2-6. Aerosol MMAD vs. Day of Exposure – Chlorotrifluoroethylene (0.25 mg/L).

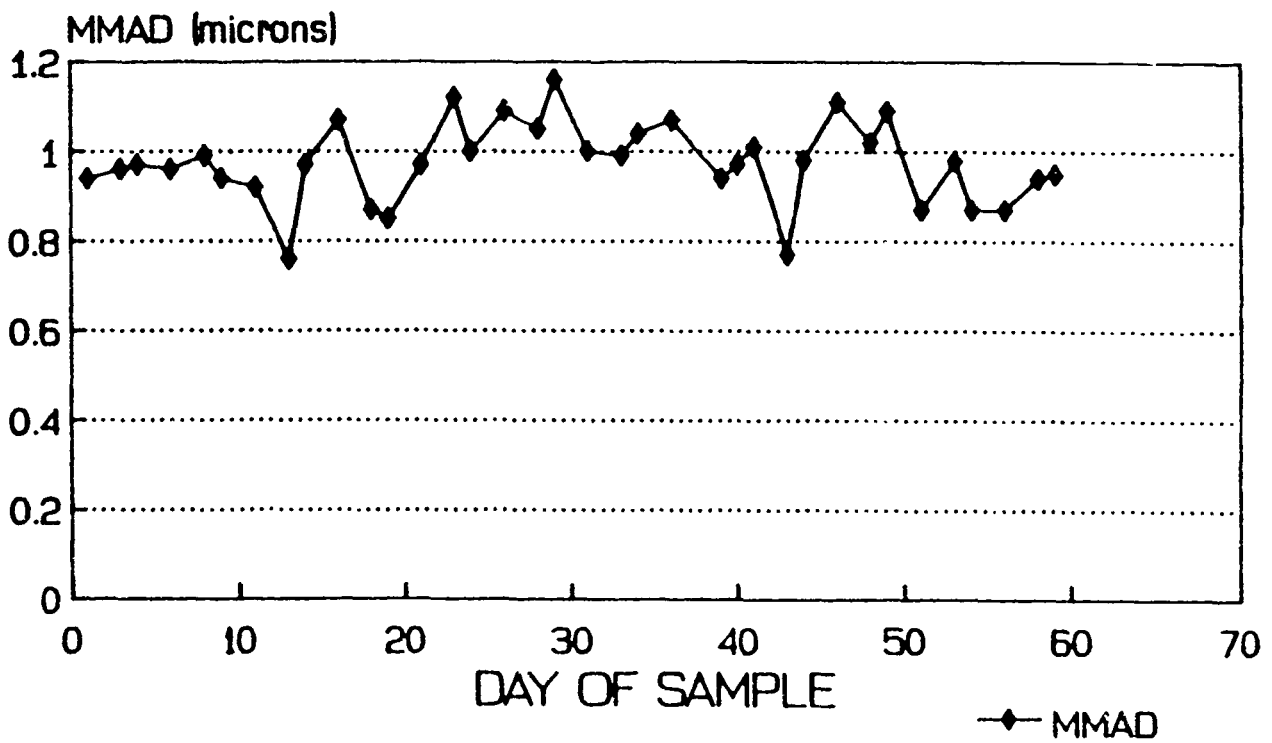

Figure 3.2-7. Aerosol MMAD vs. Day of Exposure – Chlorotrifluoroethylene (0.50 mg/L).

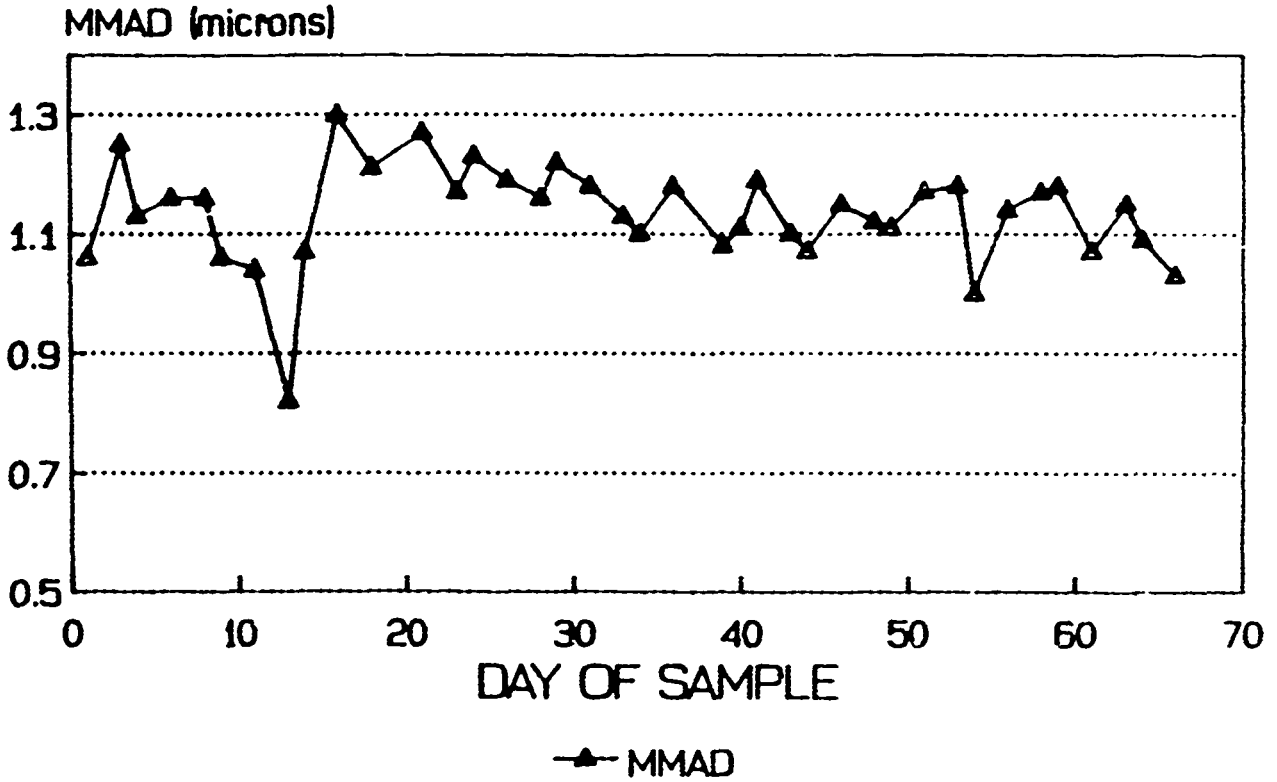

Figure 3.2-8. Aerosol MMAD vs. Day of Exposure – Chlorotrifluoroethylene (1.00 mg/L).

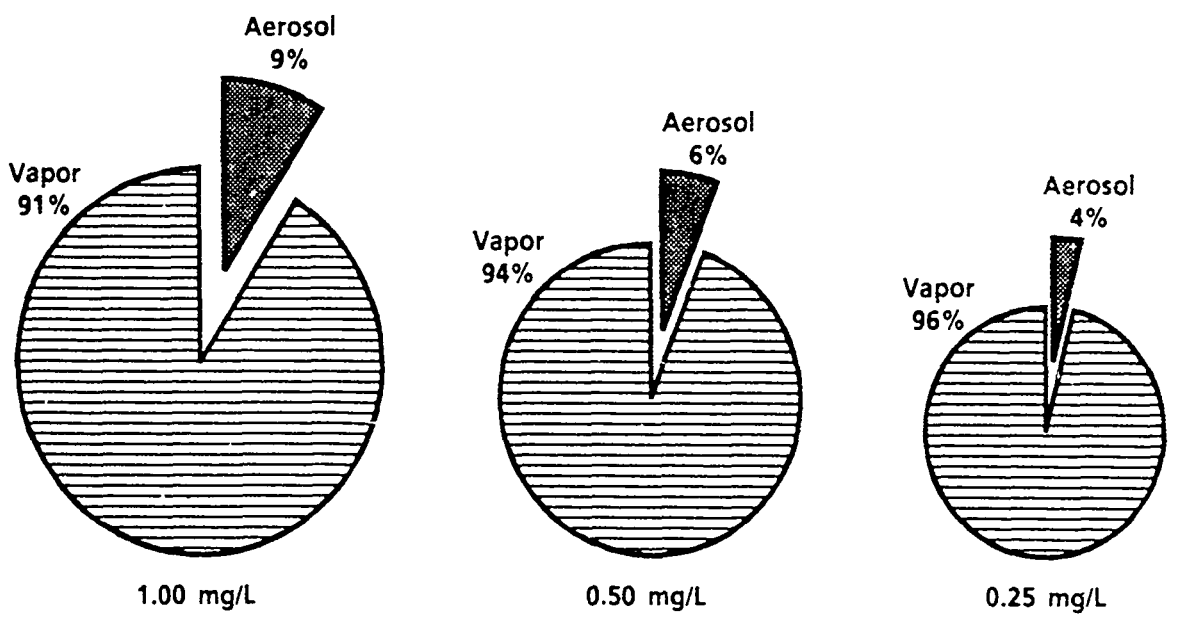

Figure 3.2-9. Ratio of Vapor to Aerosol in the CTFE Exposure Chambers.

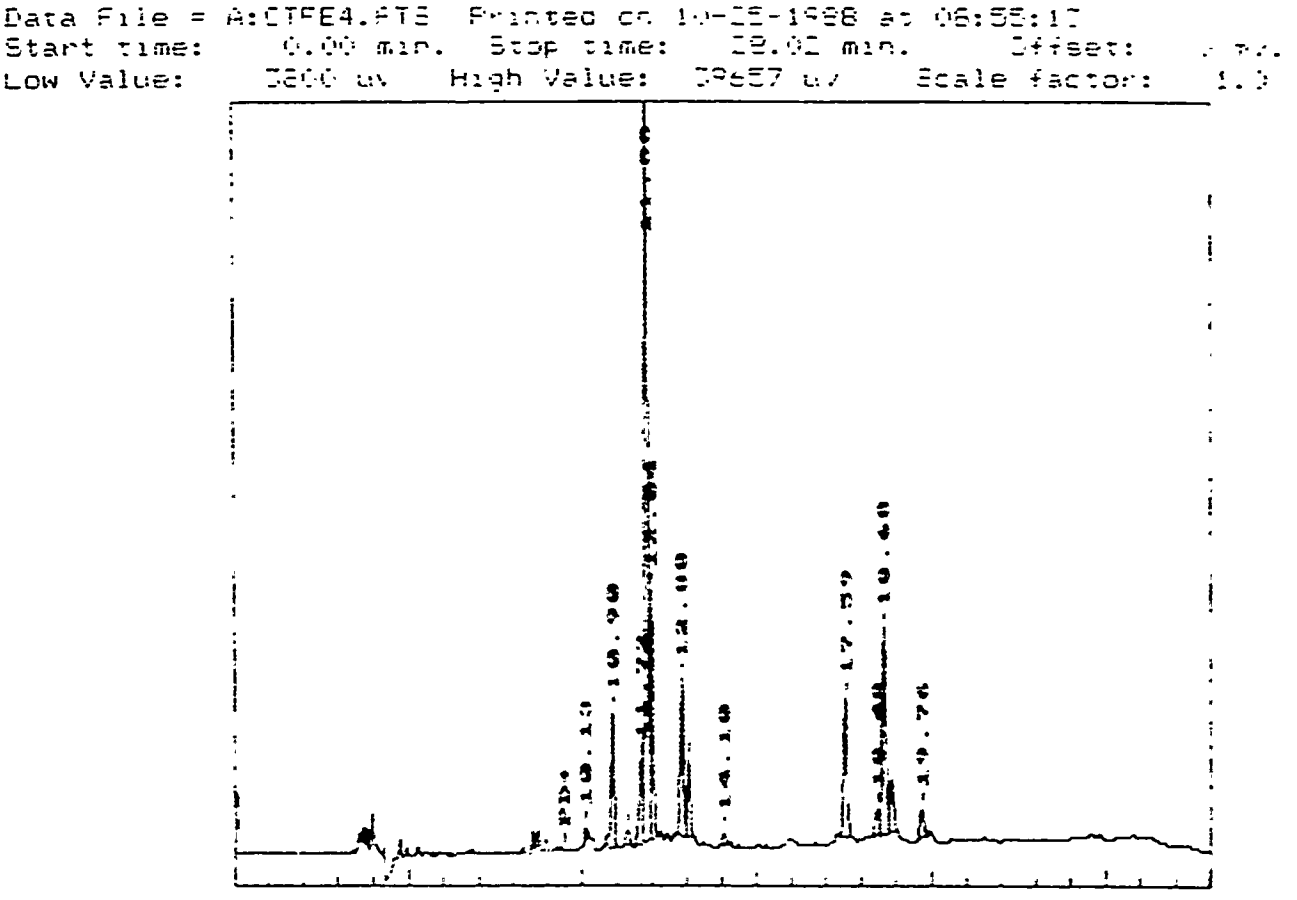

Figure 3.2-10. Typical Chromatogram Using an Electron Capture Detector, Showing Two Distinct Groups of Peaks.

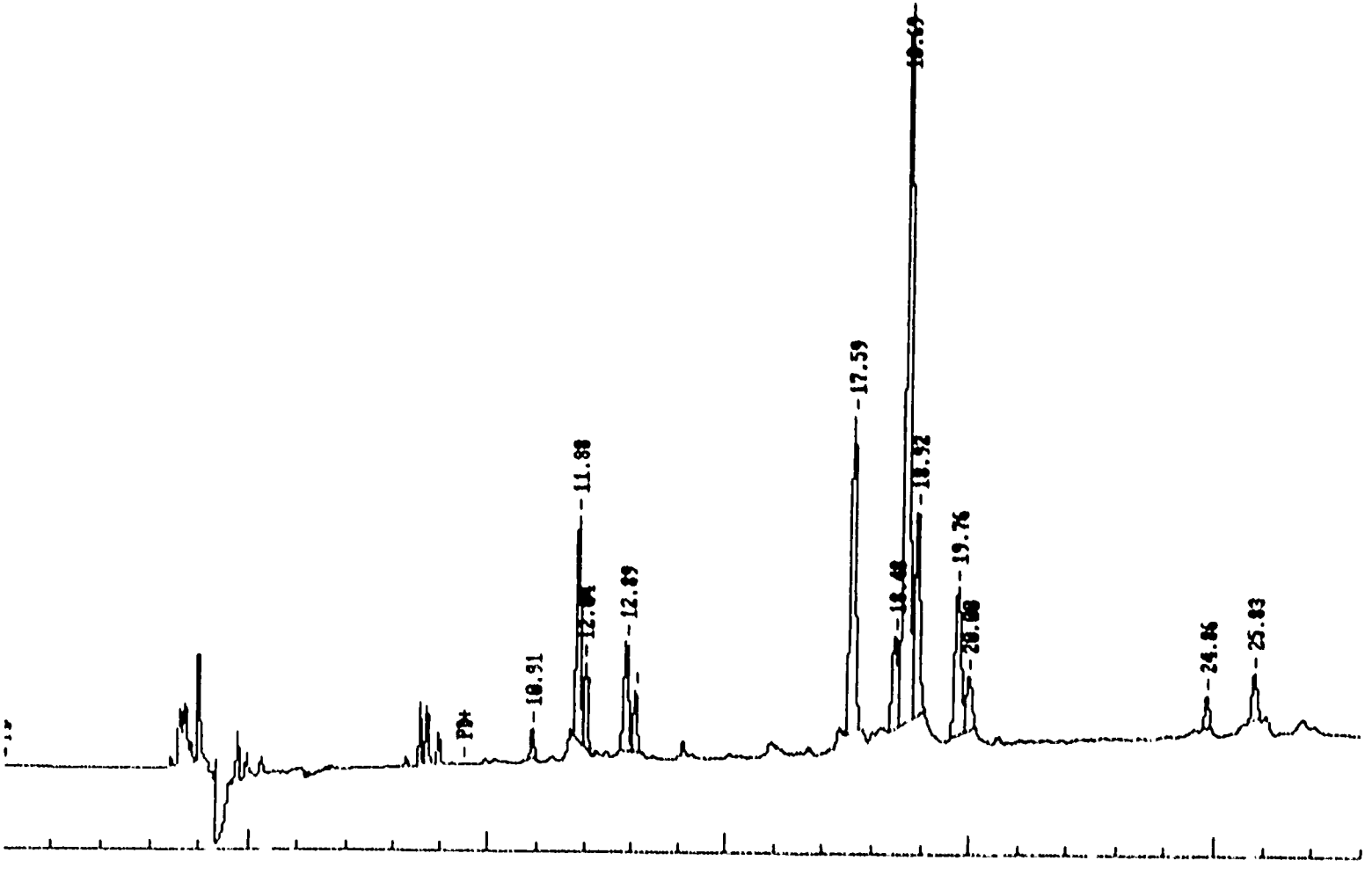

Figure 3.2-11. Typical Chromatogram of CTFE Aerosol Fraction of Exposure Chamber Atmosphere.

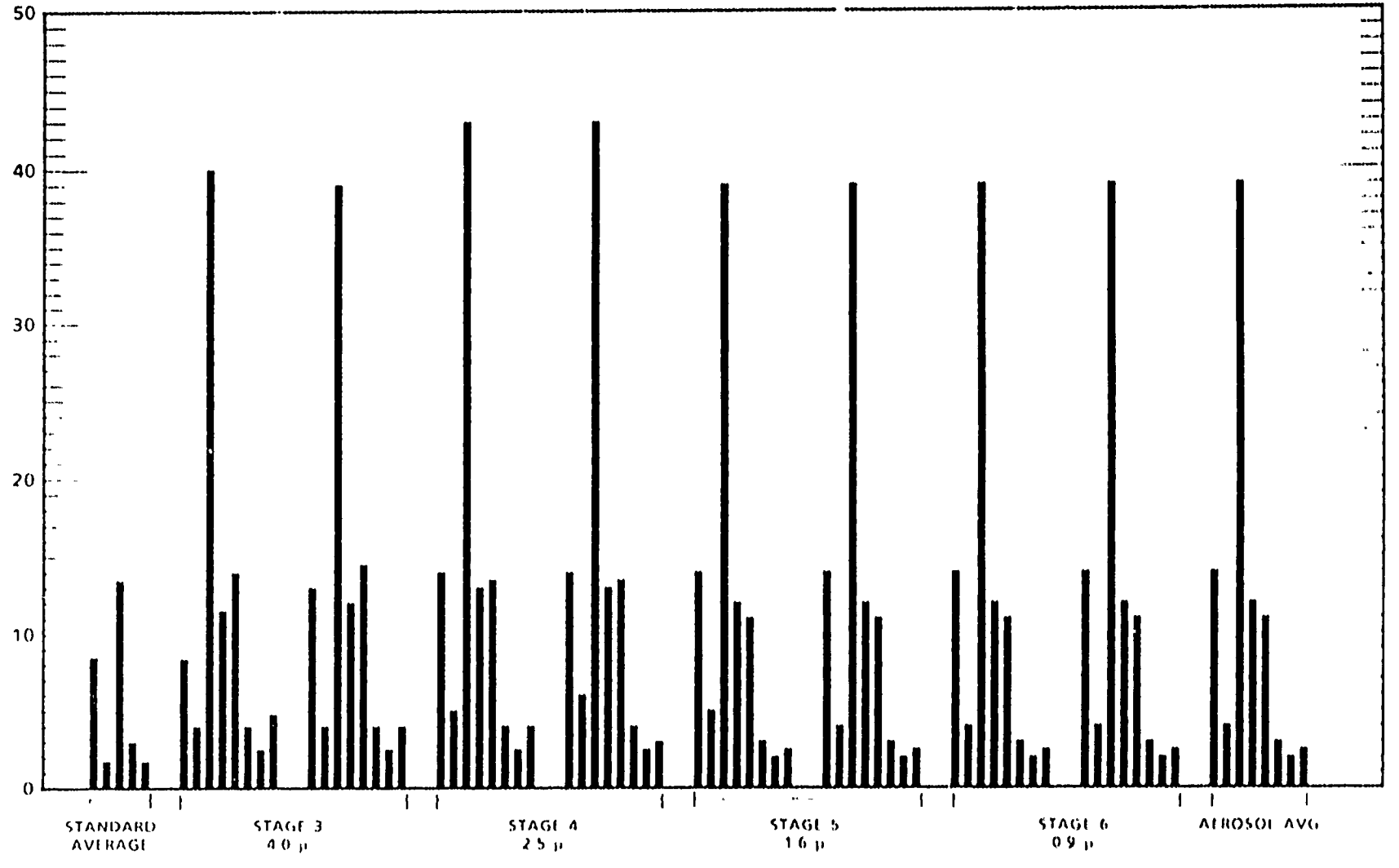

Figure 3.2-12a. Duplicate Cascade Impactor Sample Analyses. Chromatographic analysis for group 2 oligomers from each impactor stage collected from chamber atmosphere

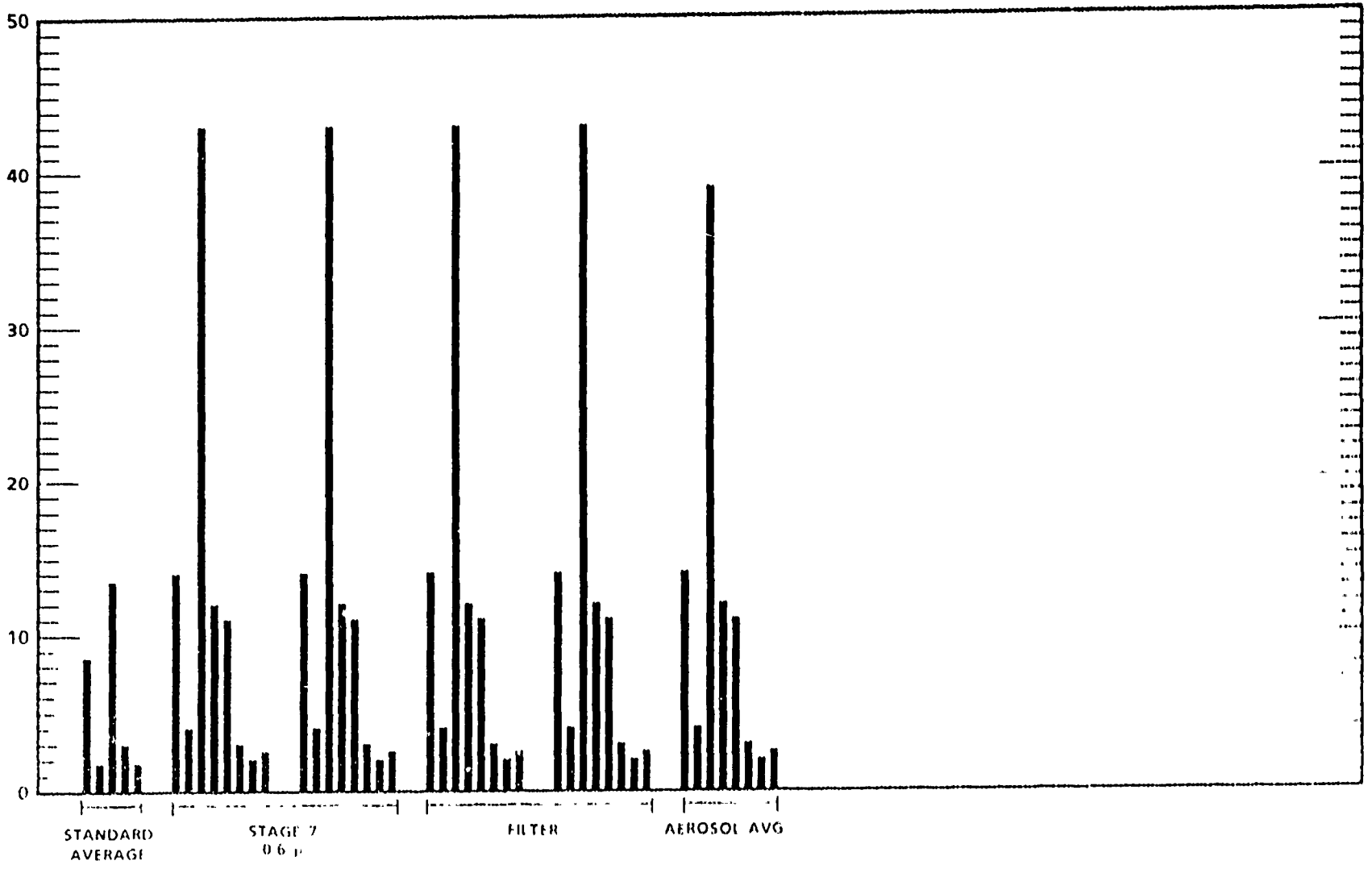

Figure 3.2-12b Duplicate Cascade Impactor Sample Analyses. Chromatographic analysis for group 2 oligomers from each impactor stage collected from chamber atmosphere

3.3 ANALYSIS OF CHLOROTRIFLUOROETHYLENE OLIGOMERS IN A MIXED AEROSOL-VAPOR SYSTEM

H.F. Leahy, J.W. Young, C.R. Doarn, and H.C. Higman

ABSTRACT

Chlorotrifluoroethylene (CTFE) oligomers were generated at three target concentrations for a 90-day inhalation study. The resulting chamber atmospheres were a mixed vapor-aerosol. The results of the characterization of these atmospheres are reported. Vapor phase components were enriched in lower molecular weight oligomers while aerosol components were enriched in the less volatile oligomers.

INTRODUCTION

CTFE is of interest to the U.S. Air Force for use as a nonflammable hydraulic fluid. The fluid is composed of oligomers of CTFE, mainly the C5 through C8 fractions. This study was designed to expose animals in inhalation chambers to vapors and aerosol of CTFE oligomer at 1.0, 0.5, and 0.25 mg/L, 6 h a day for 90 days, with no exposure on weekends.

Collison nebulizers were used to generate chamber contaminants. The consistency of the generation and high percentage of vapor in the chambers made possible continuous concentration analysis of the chamber atmospheres for CTFE using long-path infrared gas analyzers. Continuous monitoring resulted in excellent control of the generation.

Physical and chemical properties of the chamber atmospheres were characterized by both gravimetric and gas chromatographic (GC) analyses. The aerosol phase was characterized by use of filter samples for determination of aerosol mass and concentration, impactor samples for particle size distribution, and continuous photometric aerosol mass analysis to define the stability of the aerosol component. GC analyses were performed on the supply CTFE fluid, CTFE from the nebulizer reservoirs, filter extracts, solutions from the impactor stages, chamber vapor alone, and chamber vapor and aerosol for comparison of the component GC peak distribution in these various samples.

This paper describes the GC analytical methods and presents the results from analysis of chamber samples, as well as the distribution of oligomers in the vapor and aerosol phases. Results of the studies using these methods are described elsewhere in this report.

MATERIALS

Test Material

The CTFE used in this study was supplied by the U S Air Force The material was labeled as shown:

ML0-87-124
SAFETOL 3 1
HYDRAULIC FLUID
BATCH #86-134
10-24-86
P O F3360187M0335

ANALYTICAL METHODS

Introduction

The analytical scheme (Table 3 3-1) contains an overall view of the parameters monitored during the study. Whenever possible, samples were analyzed by more than one method.

TABLE 3.3-1. ANALYTICAL SCHEME

Parameter	Instrument	Determination
CTFE – supply	GC	composition
	Infrared	composition
	Mass spec	composition
CTFE – reservoir	GC	composition
Chamber vapor	Infrared	mass – indirect
	GC	composition & mass
Chamber vapor & aerosol	GC	composition
Aerosol – filters	Cahn balance	mass – direct
	GC	composition & mass
Aerosol – impactor	Cahn balance	size distribution
	GC	composition & mass

Gas Chromatography

All GC analyses for this study were performed under identical conditions using a Varian 3500 GC equipped with an electron capture detector (ECD) An autosampler was utilized to permit

24 hour operation and to enhance the reproducibility of injection and timing. All samples were diluted to a target concentration of 1 µg/mL for injection. Raw data were collected on a Nelson Data Acquisition System and processed directly from the Nelson data sets. Table 3.3-2 shows the conditions used for this study.

TABLE 3.3-2. GAS CHROMATOGRAPHIC CONDITIONS

Gas chromatograph	Varian 3500
Column:	30-m wide bore SPB-1 Capillary Glass
Carrier:	Nitrogen at 6 mL/min
Makeup gas:	Nitrogen at 20 mL/min
Column temp.:	55°C for min; then to 115°C at 15°C/min; then to 220°C at 5°C/min and hold
Injector temp.	250°C
Detector:	Electron Capture at 320°C
Injection mode:	Splitless

CHAMBER CONCENTRATION ANALYSIS

Vapor Mass

The CTFE vapor in each chamber was analyzed continuously using a Miran 1A long-path infrared analyzer. After determination of the optimum conditions for monitoring CTFE, peak wavelength setting, pathlength, and range of signal amplification, each analyzer was calibrated against a series of standards of known concentrations of CTFE vapor introduced using standard bags. Statistical best-fit curves were calculated from these data. Concentrations were then calculated directly from the best-fit curves that were validated weekly during the course of the exposure.

Aerosol Mass

The aerosol concentration in each chamber was monitored continuously by a RAM-S (a light-scattering photometric instrument) to observe the stability of concentration. Quantification of the aerosol was accomplished gravimetrically by weighing filter samples. Particle size distribution was determined using a the Lovelace Multijet Impactor. A Cahn C 31 microbalance was used for determination of weight changes.

Aerosol Component Distribution

Filters and impactor stage sets from each chamber were sampled in a manner similar to the mass analysis samples and were diluted to a concentration of 1 µL/mL. All samples were analyzed using the same GC technique as was used for the quality assurance analysis of the original samples.

Using an auto injector, a 1 µL injection of the 1 µg/mL dilution, based on the reported weight of the sample, was chromatographed Hexane blanks and CTFE standards were run between sample types, and all samples were injected in duplicate

Chamber Component Distribution

Syringe samples (10, 20, and 40 mL) were used to trap a known volume of chamber atmospheres that were diluted in hexane to approximate a concentration of 1 µg/mL. These samples were then chromatographed using conditions identical to those used in the standards and aerosol analyses.

Data Storage

The original chromatography data in the form of the three Nelson files for each chromatogram were stored on mini-floppy disks, and a hard copy of each chromatogram was stored on file with the notebooks. The data were reduced for comparison studies using the Lotus 1-2-3, Version 2 spread sheet.

Quality Control

Samples were taken for analyses from each of the five supply containers An infrared spectrum of a thin layer of CTFE between salt plates was obtained using a Beckmann Acculab 4 Infrared spectrophotometer Gas chromatograms of CTFE diluted to 1 µg/mL were acquired using a Varian 3500 GC equipped with an ECD Data were obtained for each of the containers No differences were observed in the five samples by either method of analysis Table 3 3-3 summarizes data for replicate GC analyses of the stock supply samples.

RESULTS

Chamber Concentration

The chamber concentrations were computed from data supplied to the chamber data acquisition system either directly, as for the Miran 1A readings, or indirectly, as for the filter and impactor samples A summary of these data is shown in Table 3 3-4 The percentage of aerosol in the chambers increased from 4% at the 0 25 mg/L target concentration to 6% at 0.50 mg/L, then to 9% at the 1.0 mg/L

Chamber Atmosphere CTFE Component Distribution

Figures 3 3-1 through 3.3-3 illustrate the vapor and total chamber atmosphere compositions for the 0 25 mg/L, 0 50 mg/L, and 1 0 mg/L chambers as compared to the stock material Samples of each chamber atmosphere were analyzed by GC, providing comparisons of the composition of the total contaminant, vapor and aerosol, to both the vapor phase alone and also to the original CTFE

TABLE 3.3-3. SUMMARY OF REPLICATE GC ANALYSES OF SUPPLY CTFE, MLO 87-124

Peak ID	Mean Peak Areas						
Rt Min[a]	Supply #1 0872591	Supply #2 0872592	Supply #3 0872593	Supply #4 0872594	Supply #5 0872595	Mean of 5 Pairs	% Total Area
10.13	3,145	5,890	4,849	6,326	6,350	5,312	1
10.90	42,059	42,853	44,922	46,000	46,107	44,388	6
11 32	6,558	6,615	6,969	7,174	7,235	6,910	1
11 72	31,114	32,232	33,440	35,046	35,461	33,459	5
11.88	250,127	251,298	261,376	268,570	266,369	259,548	36
12.04	75,945	76,251	79,875	81,096	80,245	78,682	11
12.88	58,113	58,450	61,371	63,585	62,909	60,886	8
13.08	26,065	26,116	27,509	28,307	28,384	27,276	4
14.10	3,326	3,051	3,249	3,600	3,661	3,377	0
17.59	56,926	56,623	59,172	61,745	61,275	59,148	8
18.48	12,326	12,221	12,795	13,284	13,602	12,846	2
18.68	91,123	89,944	94,413	98,451	96,596	94,105	13
18.91	24,753	24,115	26,272	26,756	26,780	25,735	4
19.76	12,131	12,034	12,512	13,172	13,041	12,578	2
Total Area	693,712	697,694	728,722	753,110	748,019	724, 251	

[a] Rt Min = Retention time in minutes

TABLE 3.3-4. DATA SUMMARY OF THE CHAMBER CONCENTRATION OF CTFE

	Chamber		
	0.25 mg/L	0.50 mg/L	1.00 mg/L
Vapor mg/L	0 24 96%	0 45 94%	0.89 91%
Aerosol mg/L	0.01 4%	0 03 6%	0 09 9%
Total mg/L	0 25	0 48	0 98
% Aerosol	5 3	6 8	8 85
MMAD[a]	1 20	0 97	1.14
GSD[b]	2 59	2 15	2.17

[a] MMAD = Mass Medium Aerodynamic Diameter
[b] GSD = Geometric Standard Deviation

Figures 3 3-4 and 3 3-5 depict the measurable differences in the composition of the atmospheres (vapor alone or vapor and aerosol) in each of the chambers, and show that these were concentration-dependent. The vapor alone showed an increased percentage of the more highly volatile materials

Figures 3 3-6 through 3 3-8 depict the differences in the distribution of CTFE oligomers for the total atmospheres as compared to the stock CTFE These differences were slight and show that the overall concentration in the chambers was representative of the original stock material

Nebulizer CTFE Component Distribution

Figure 3.3-9 depicts the concentration of CTFE oligomers remaining in the nebulizer after generation for each of the three chambers CTFE remaining in the nebulizer reservoirs was analyzed to define distillation effects related to nebulization Some loss of the more volatile components resulted in an increased concentration of less volatile components. The disproportionation of oligomeric distribution was chamber concentration related, with the highest concentration chamber demonstrating the greatest change

Aerosol CTFE Component Composition

Figure 3.3-10 shows the distribution of CTFE oligomer in the aerosol sampled from the chambers as determined from the GC peak area distribution both from filter extracts and washings from impactors. The aerosol composition appeared different from the parent material The more volatile CTFE oligomers were almost totally vaporized, leaving an increased percentage of less volatile oligomers in the aerosol phase. Analysis of the aerosol data demonstrated that there were several observable trends related to the concentration.

Figures 3.3-11 through 3 3-13 illustrate that the oligomer content of the aerosol was dependent on the concentration generated The highest relative percentage of the less volatile materials (those with retention times greater than 15 minutes) was found in the aerosol from the lowest concentration chamber, whereas very little aerosol was found in the test atmosphere Almost all of the more volatile components in the low concentration chamber existed as vapor In the 0 50- and 1 00-mg/L chambers, more aerosol was found, and a greater relative percentage of this aerosol was the volatile components (retention times less than 15 minutes)

Figures 3.3-14 through 3.3-16 illustrate that the distribution of oligomers found in the aerosol was similar in aerosol particles of different sizes The data presented in these figures represent those plates of the cascade impactor where most of the aerosol mass had been deposited Such uniform oligomer composition in aerosols of differing size should result in rather consistent oligomer deposition throughout the lung regions affected by the aerosols

These data indicate that while the overall composition of the aerosol phase is dependent on the concentration generated, the composition of the aerosol does not vary with the size of the particles.

INDUSTRIAL HYGIENE

The industrial hygiene analysis for CTFE was performed using the Miran 980 infrared analyzer. Baseline exposure laboratory air was established using a wavelength at which CTFE does not absorb This reading was compared to that at a wavelength at which CTFE absorbs strongly. Standard bags containing known concentrations of CTFE were used to calibrate the analyzer The minimal detectable limit was 1 µg/L. No excursions due to loss of CTFE from chambers to the atmosphere were observed during the study

DISCUSSION

Consistent and reproducible analytical methods have been developed for chamber monitoring and analysis of vapor and aerosol phases of CTFE and applied to the characterization of chamber atmospheres generated during a 90-day exposure of animals to the material The overall composition, vapor-aerosol distribution, and oligomeric content of the vapor and aerosol phases have been defined and quantified. Data from this study will be utilized to model the pharmacokinetics of CTFE in rats and develop predictive models to help design further studies.

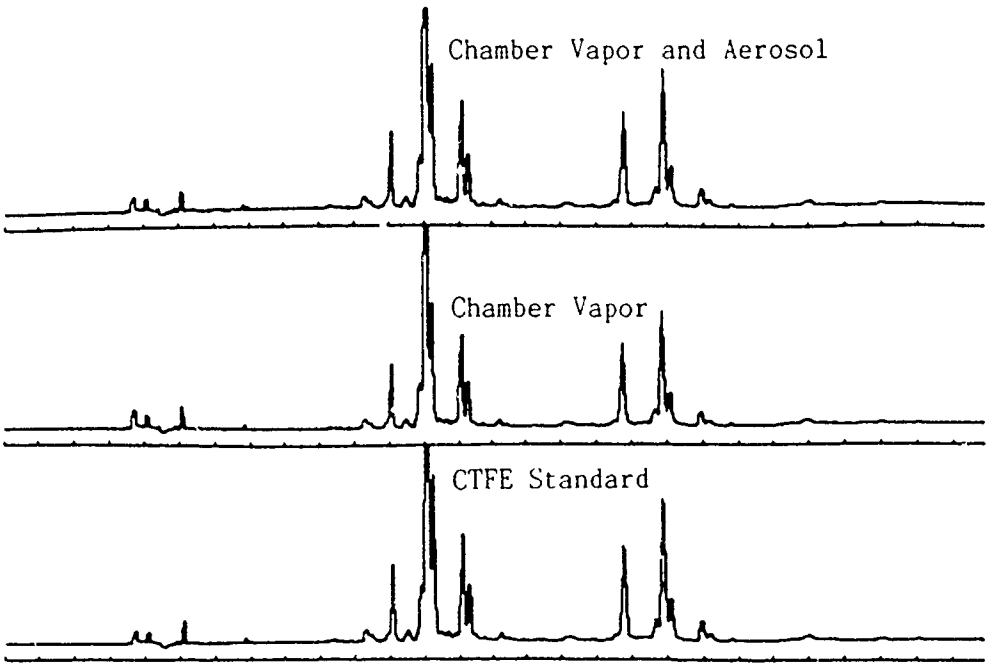

Figure 3.3-1. **Comparison of Chamber Atmosphere to Stock CTFE for 0.25 mg/L Chamber.** Gas chromatograms were obtained using electron capture detector (ECD) with dilutions to 1 µg/mL of samples and standards

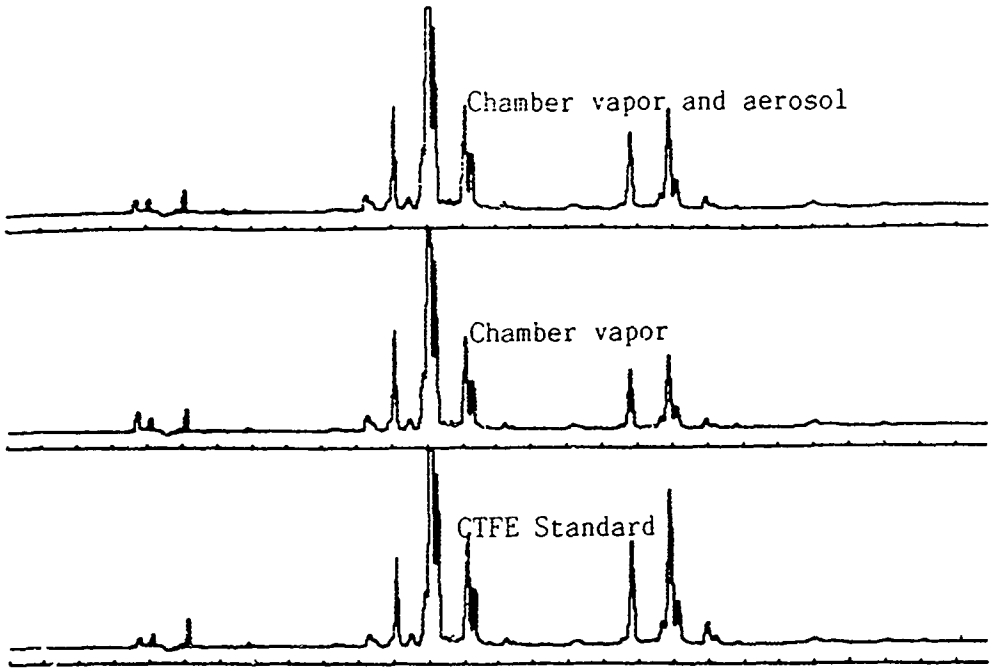

Figure 3.3-2. **Comparison of Chamber Atmosphere to Stock CTFE for 0.50 mg/L Chamber.** Gas chromatograms were obtained using electron capture detector (ECD) with dilutions to 1 µg/mL of samples and standards.

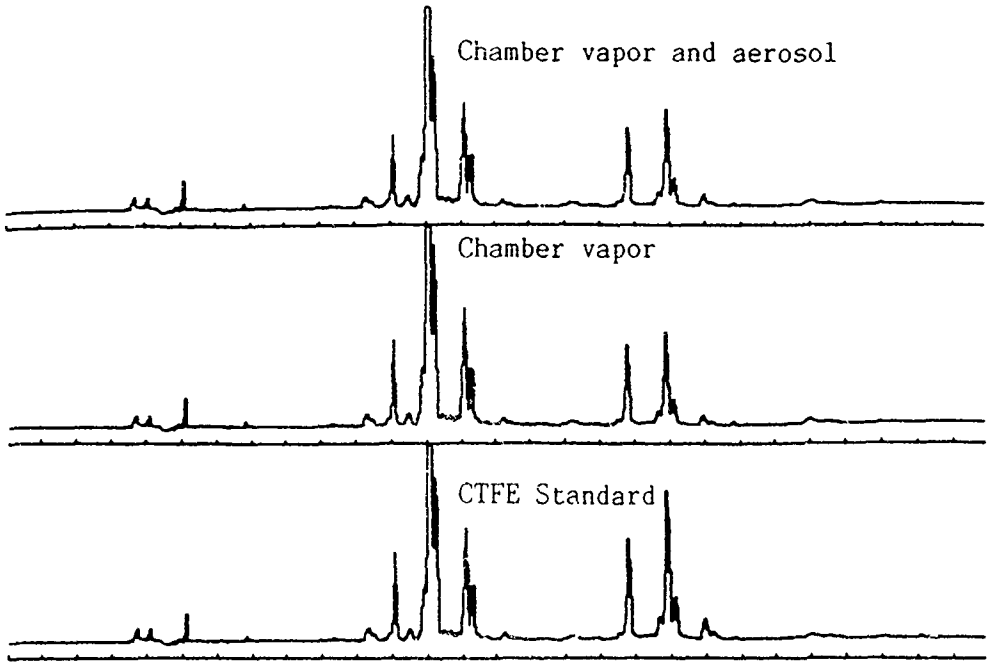

Figure 3.3-3. **Comparison of Chamber Atmosphere to Stock CTFE for 1.0 mg/L Chamber.** Gas chromatograms were obtained using electron capture detector (ECD) with dilutions to 1 µg/mL of samples and standards.

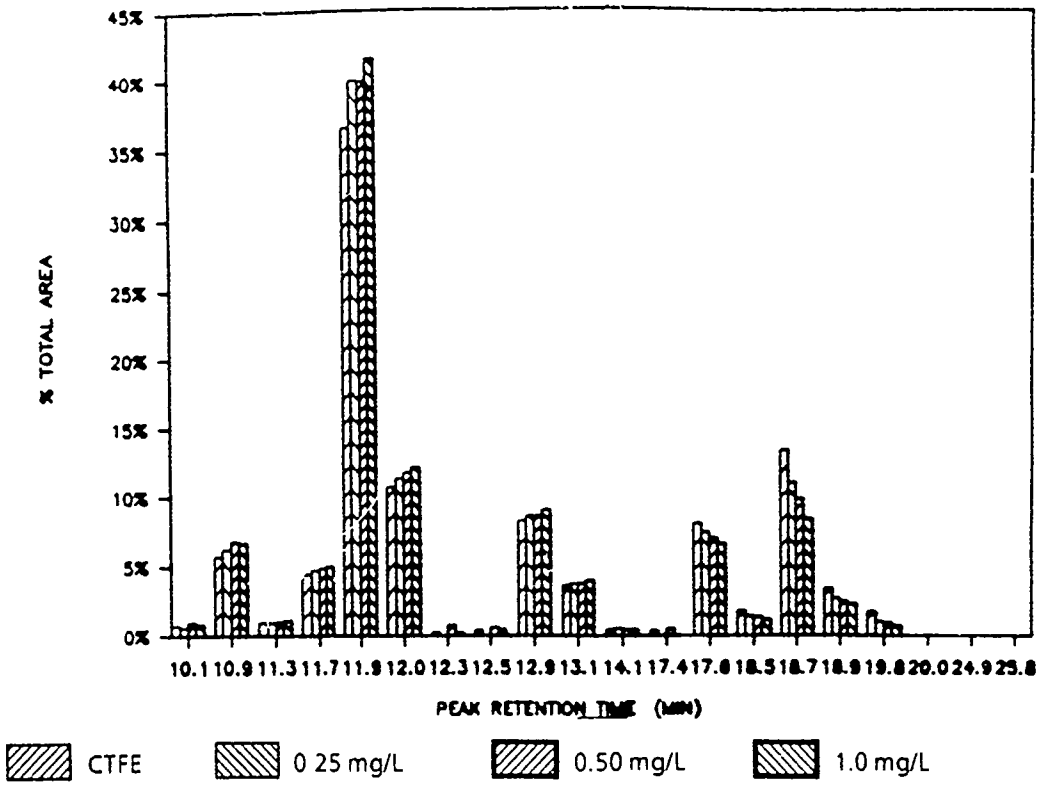

Figure 3.3-4. Distribution of Vapor Phase CTFE Oligomers in Chambers Compared to Stock CTFE.

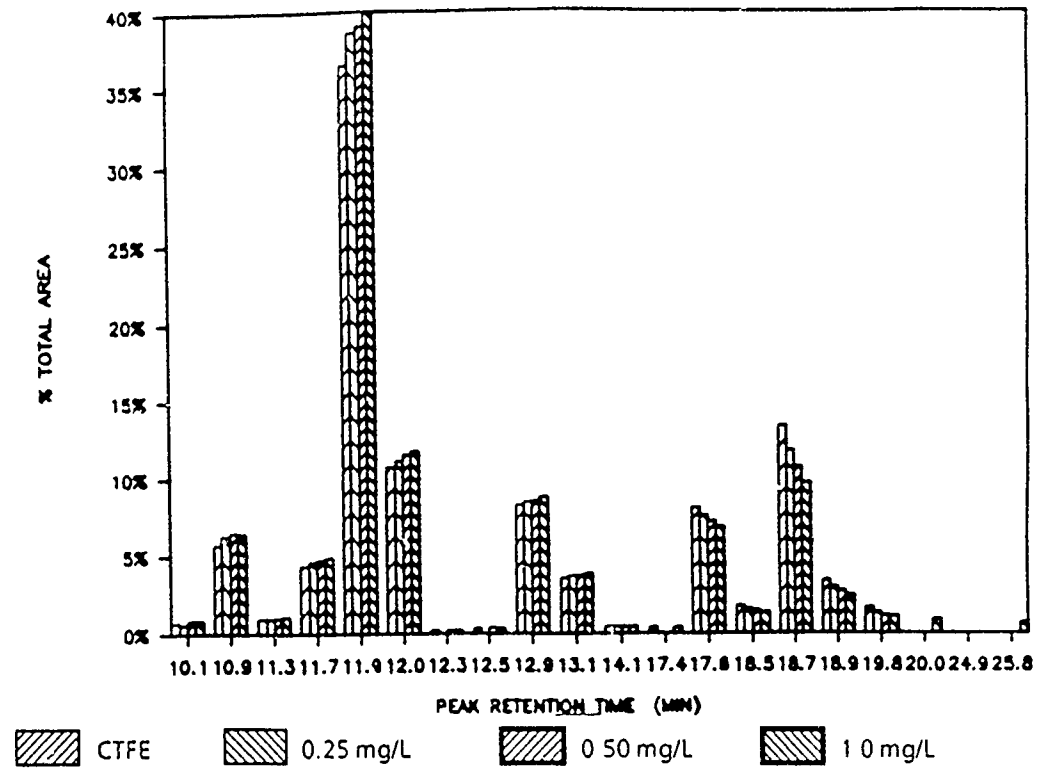

Figure 3.3-5. Distribution of Vapor Phase and Aerosol Components in Chambers Compared to Stock CTFE.

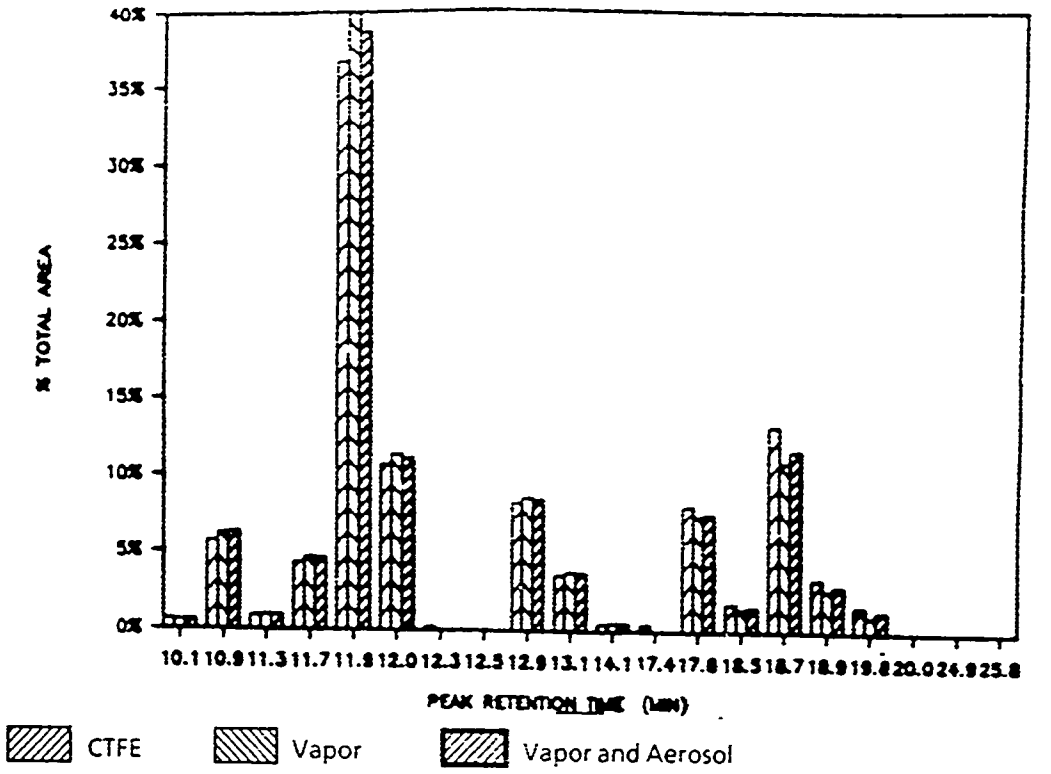

Figure 3.3-6. Comparison of Chamber Vapor and Vapor Plus Aerosol Compositions in the 0.25 mg/L Chamber to Stock CTFE.

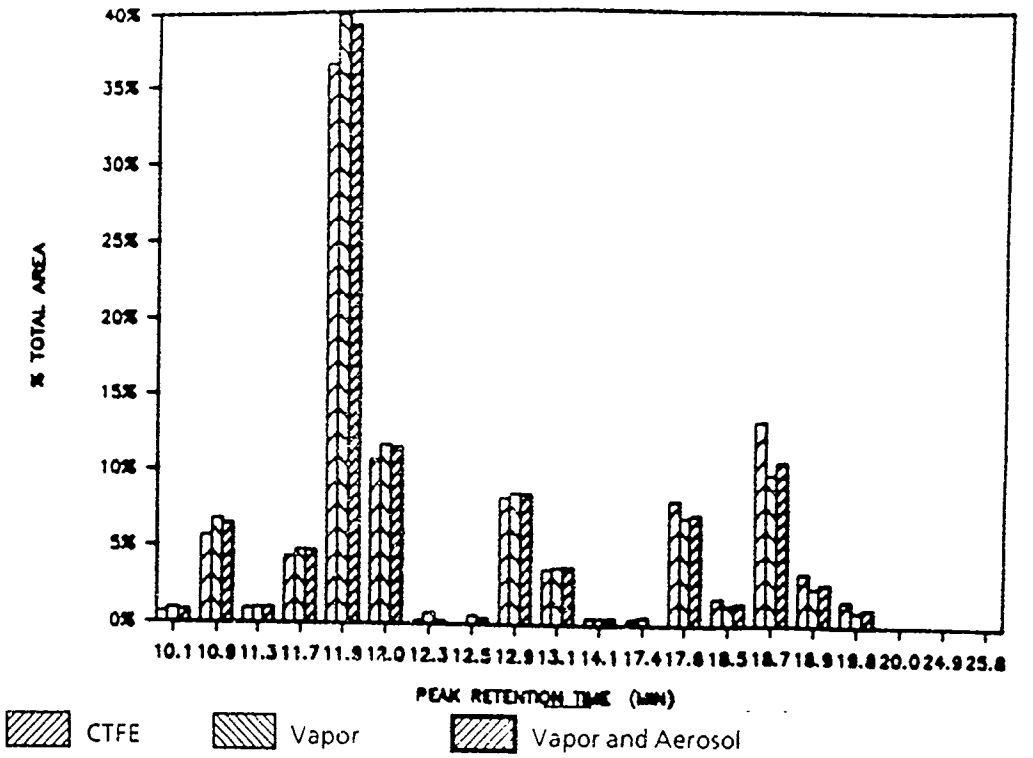

Figure 3.3-7. Comparison of Chamber Vapor and Vapor Plus Aerosol Compositions in the 0.50 mg/L Chamber to Stock CTFE.

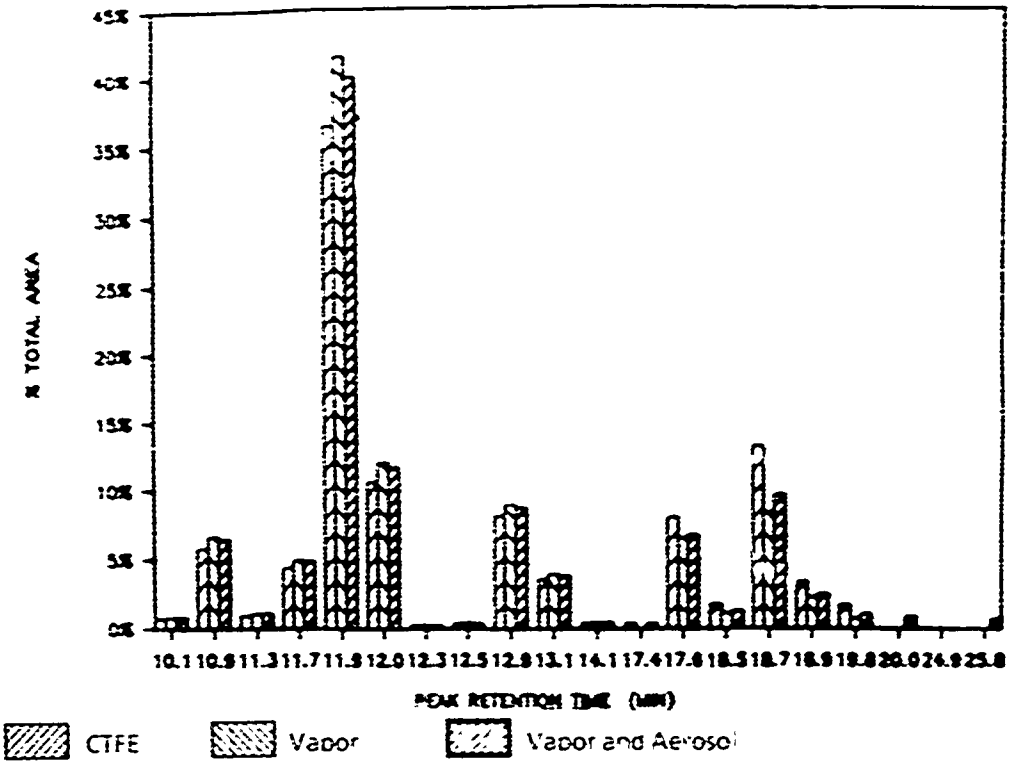

Figure 3.3-8. Comparison of Chamber Vapor and Vapor Plus Aerosol Compositions in the 1.0 mg/L Chamber to Stock CTFE.

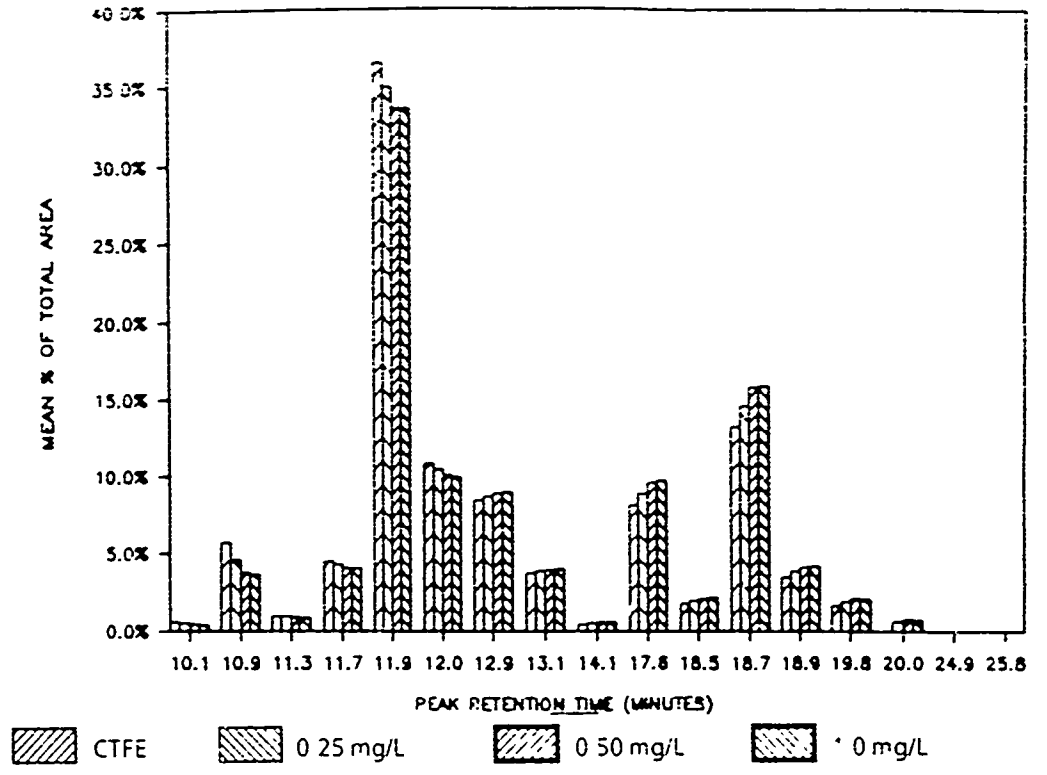

Figure 3.3-9. Comparison of the Distribution of CTFE Oligomers in Nebulizer Reservoirs after Generation Compared to Stock CTFE.

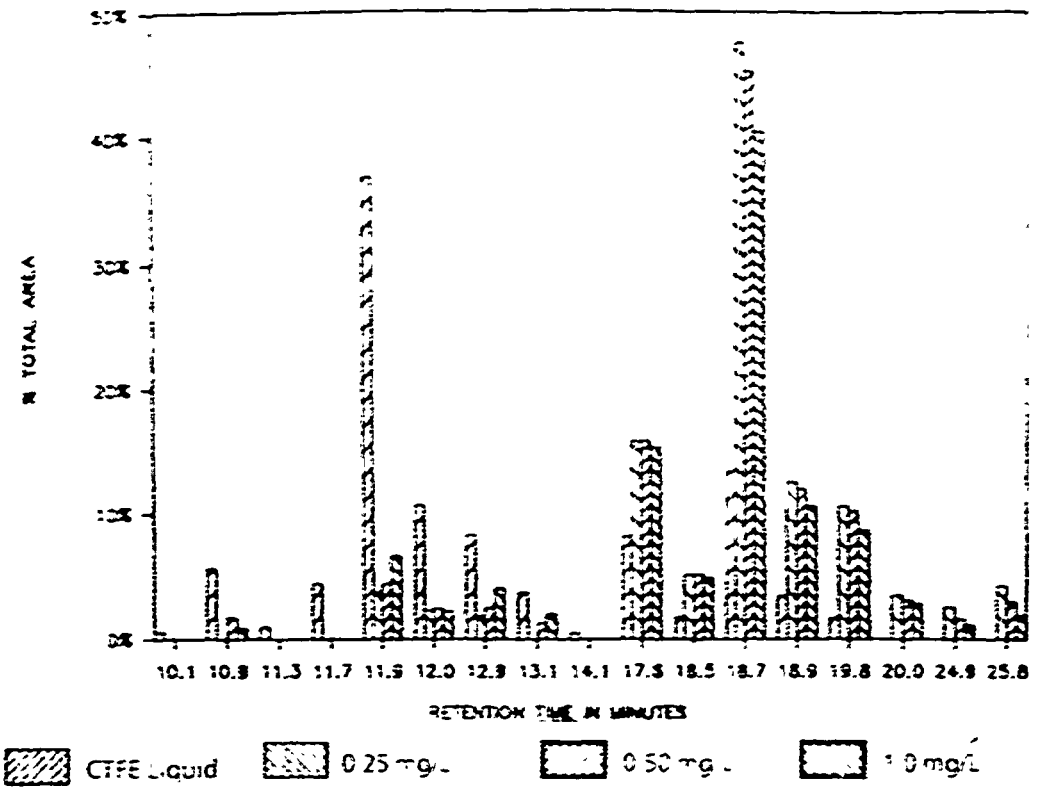

Figure 3.3-10. Distribution of Oligomers of CTFE from Filter Catch of Aerosols in Exposure Chambers as Compared to Stock CTFE.

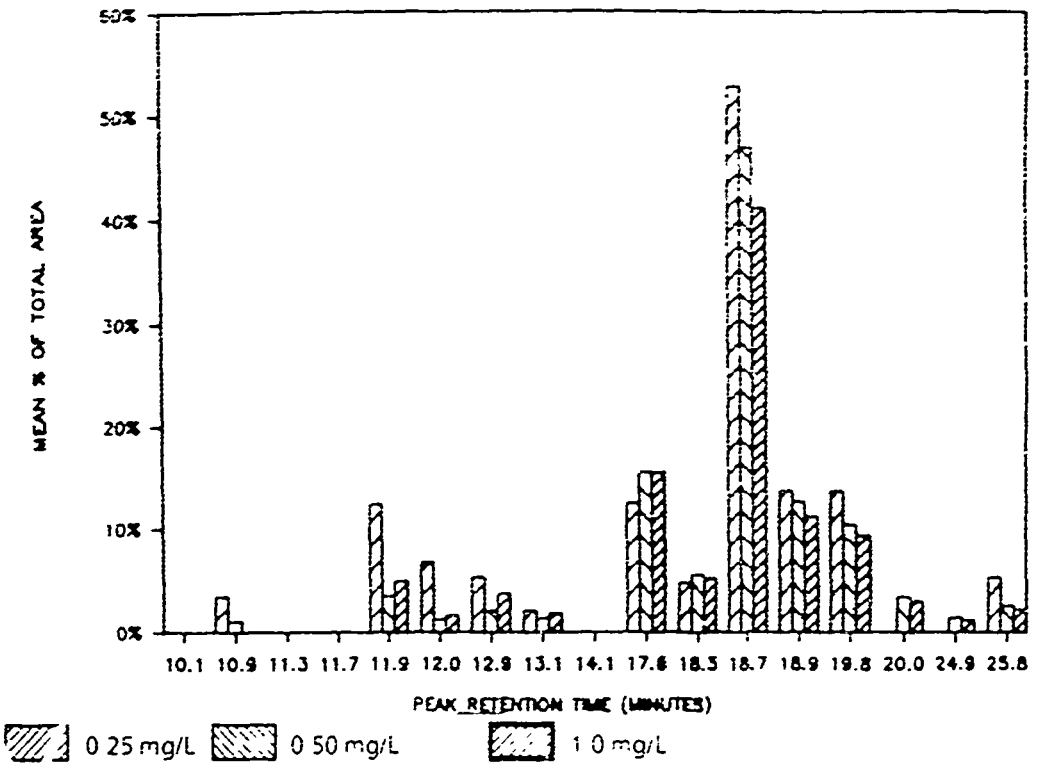

Figure 3.3-11. Distribution of CTFE Oligomers from Impactor Stage 7 for the Exposure Chambers. (Stage 7 is smallest diameter particles.)

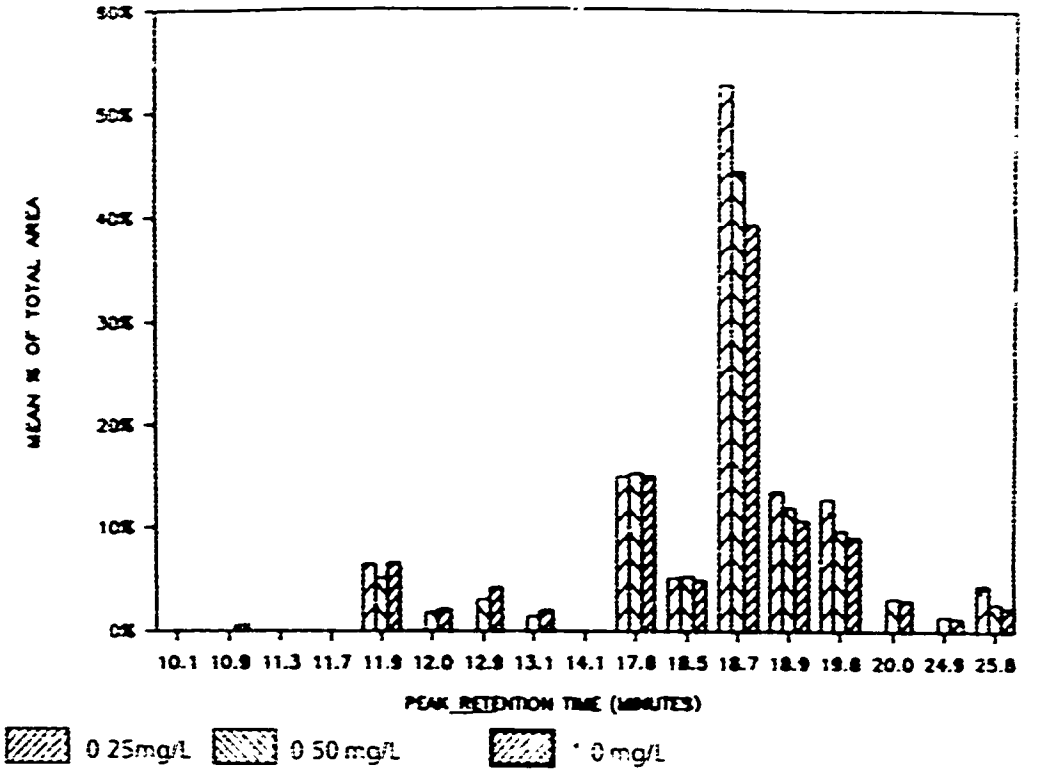

Figure 3.3-12. Distribution of CTFE Oligomers from Impactor Stage 6 for the Exposure Chambers.

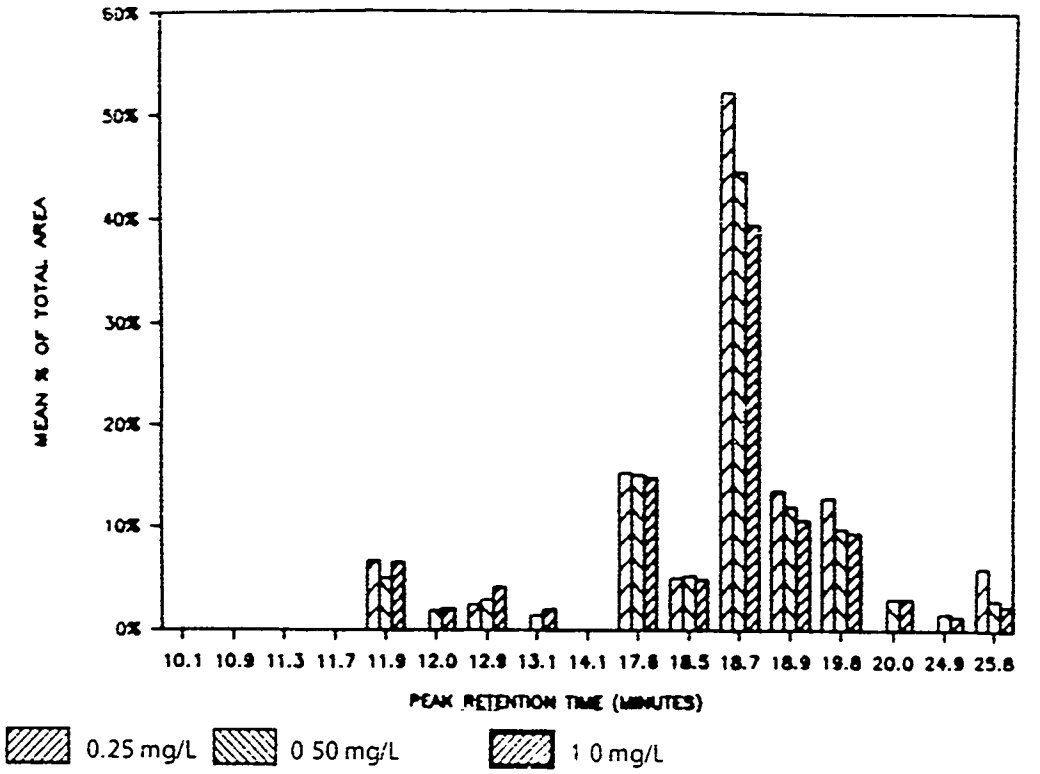

Figure 3.3-13. Distribution of CTFE Oligomers from Impactor Stage 5 for the Exposure Chambers.

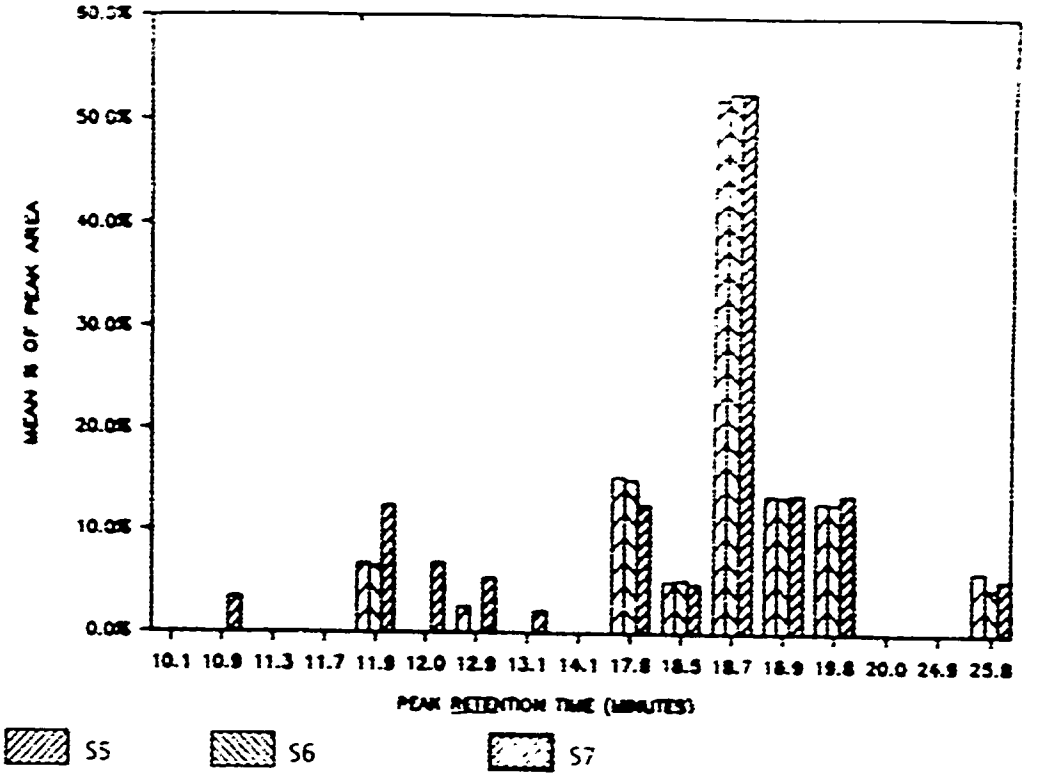

Figure 3.3-14. Comparison of the CTFE Oligomer Distribution for Impactor Stages 5, 6, and 7 from the 0.25 mg/L Chamber.

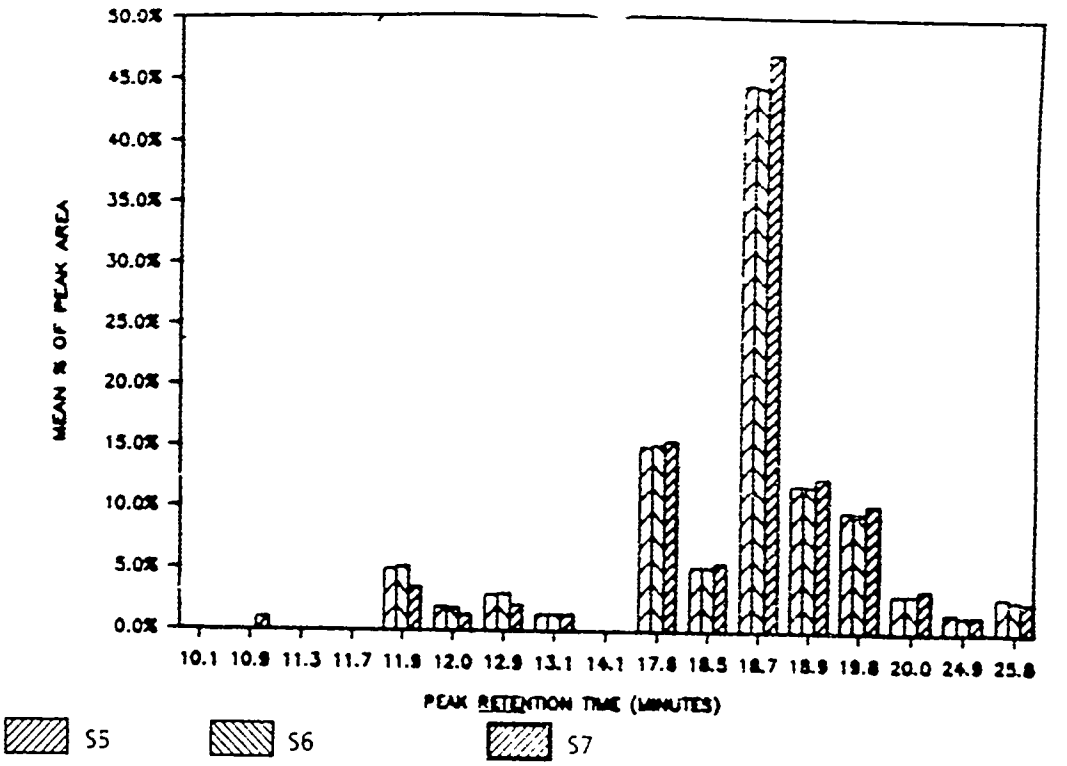

Figure 3.3-15. Comparison of the CTFE Oligomer Distribution for Impactor Stages 5, 6, and 7 from the 0.50 mg/L Chamber.

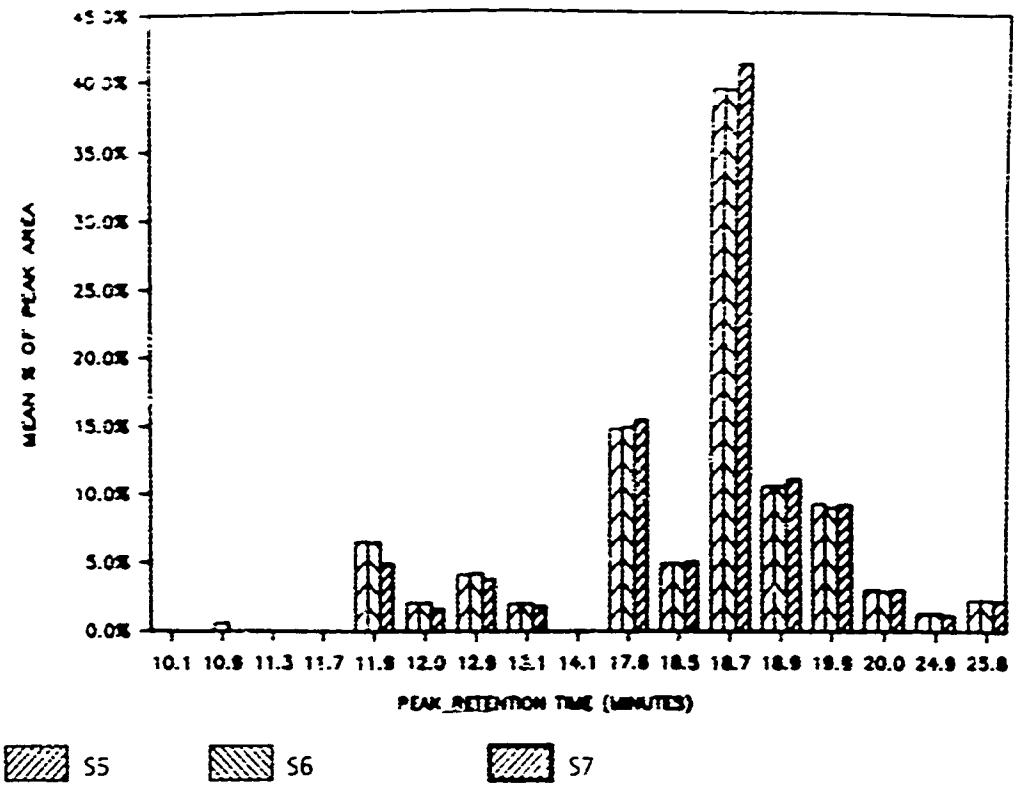

Figure 3.3-16. Comparison of the CTFE Oligomer Distribution for Impactor Stages 5, 6, and 7 from the 1.0 mg/L Chamber.

3.4 ANALYSIS OF CHLOROTRIFLUOROETHYLENE OLIGOMERS IN TISSUE, BLOOD, AND URINE

D.L. Pollard and H.C. Higman

ABSTRACT

Analytical methods for the analysis of chlorotrifluoroethylene (CTFE) in tissue, blood, and urine have been developed These procedures were used to monitor the distribution and clearance of CTFE from animals exposed by inhalation for 90 days Tissues from animals exposed for one day were also analyzed to provide additional deposition and clearance data.

INTRODUCTION

CTFE oligomer is an oil that shows promise as a hydraulic fluid CTFE is nonflammable and noncorrosive Its physical properties – high thermal stability, high dielectric strength, and good lubricity – are desirable also Recent dermal and inhalation studies indicate that CTFE has a low degree of toxicity (Gargus, 1983; Coate, 1984, Kinkead et al , 1987) Subchronic inhalation studies were designed to determine the no observable-effect level, to determine chronic exposure criteria, and to identify possible target organ toxicity. Three groups of test rats were exposed to concentrations of 1.0, 0.5, and 0.25 mg CTFE/L for 90 days over a 6-h exposure period each day

A parallel inhalation study was conducted to develop a physiologically based pharmacokinetic model for CTFE. The 0.5 mg/L chamber contained animals for use in pharmacokinetic studies. At the conclusion of the 90-day study two groups of age-matched animals were exposed to 0 5 mg/L of CTFE for one day. The analysis of biological samples and tissues for CTFE is the subject of this report.

Biological samples were taken from both the 6-h exposed rats and inhalation study animals exposed for 90 days for use in the development and testing of the model. Seven groups of four rats each were used in this portion of the study for determination of CTFE tissue concentrations The groups are designated as follows

- Three groups of test rats were exposed 6 h per day, five days per week, to a 0.5 mg CTFE/L target concentration for 90 days Each group was removed from the exposure for sampling near the end of the 90-day exposure
- Two groups of control rats were exposed one day for 6 h, to a 0 5 mg CTFE/L target concentration
- Two groups were control

Blood was taken at intervals following removal from CTFE exposure for CTFE analysis Urine and feces were collected for CTFE analysis from each animal group Kidney, lung, liver, testes, brain, and fat were taken at sacrifice for CTFE analysis

MATERIALS

Test Agent

Halocarbon oils are saturated, low molecular weight polymers of CTFE with the general formula $(CF_2CFCl)_n$ They are made with a controlled polymerization technique using chlorine to end cap the polymers The resulting oligomeric mixture is chemically stable and inert. Fractions containing sets of oligomers are separated by vacuum distillation to provide desired physical properties The designation 3 1 for the study material defines a viscosity of 3 1 for the oligomeric mixture.

CTFE was supplied by the U S Air Force Standards were prepared from the CTFE taken from a plastic container of approximately five gallons and labeled as shown.

MLO 87-124
SAFETOL® 3.1
Hydraulic Fluid
Batch # 86-134
10-24-86
P O. F3360186M0335

Test Material Analysis

CTFE was analyzed to determine the major component retention times and elution patterns by gas chromatography using two types of detectors Electron capture detection (ECD), which is extremely sensitive to halogenated materials, was used to determine the elution pattern and relative percentage peak areas A thermal conductivity detector (TCD) also was used to verify that the percent area values obtained with the ECD were directly related to the concentration of the oligomers, since ECD detectors often respond in a non-linear fashion. The TCD analysis verified that the values obtained from ECD analysis could be used directly to calculate concentration and relative percent areas

Table 3.4-1 shows the conditions used for the electron capture analysis of CTFE These conditions were used throughout the study for blood, urine, feces, and tissue analysis Identical flow and temperature conditions were employed for the TCD analysis

TABLE 3.4-1. GAS CHROMATOGRAPHIC CONDITION FOR ELECTRON CAPTURE CTFE ANALYSIS

Gas chromatograph	Varian 3700
Column	75MM ID x 30M OD SPB-1
Column temperature	70°C - 2°C/min - 130°C
Injector temperature	150°C
Detector	Electron capture
Detector temperature	300°C
Helium carrier flow	10 3 mL/min
Argon/methane make-up flow	25 mL/min

Assessment of Chromatographic Data

The chromatogram shown in Figure 3.4-1 illustrates the distribution of CTFE oligomers using the conditions in Table 3.4-1. There are two discrete groups of peaks, labeled Group A and Group B on the chromatogram. The Group A sets of peaks are more volatile and of lower molecular weight than the peaks in Group B. Analysis of exposure atmospheres demonstrated that the Group A peaks were almost completely vapor, while the peaks in Group B were distributed between the vapor and aerosol phases. The aerosol phase was composed of Group B components.

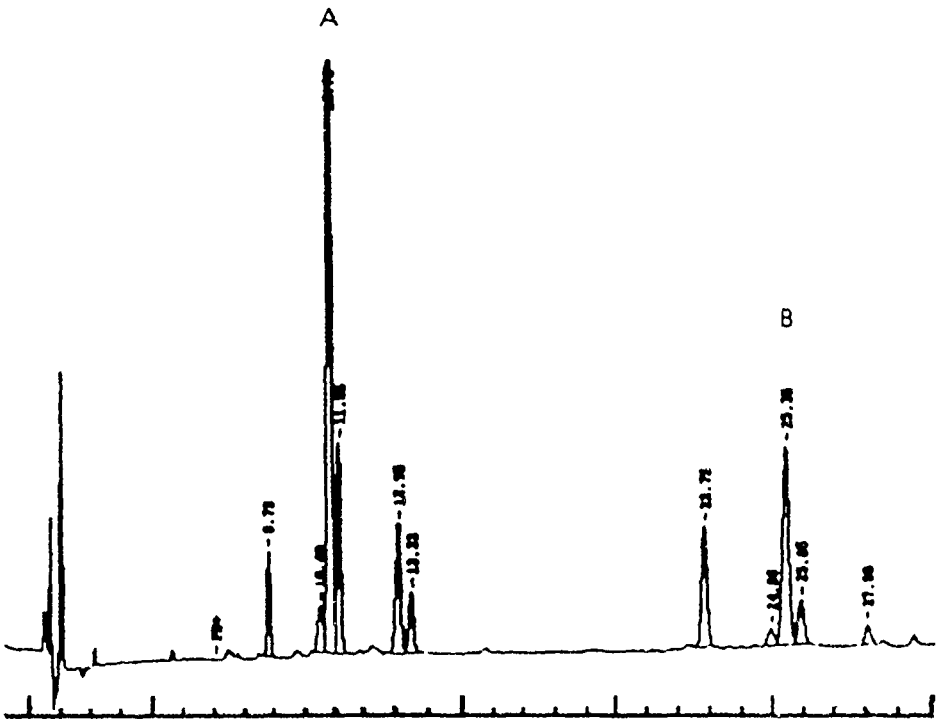

Figure 3.4-1. ECD Gas Chromatogram of CTFE.

Figure 3 4-2 shows the TCD chromatogram for comparison to the ECD data. The distribution is similar in percent area using both detectors

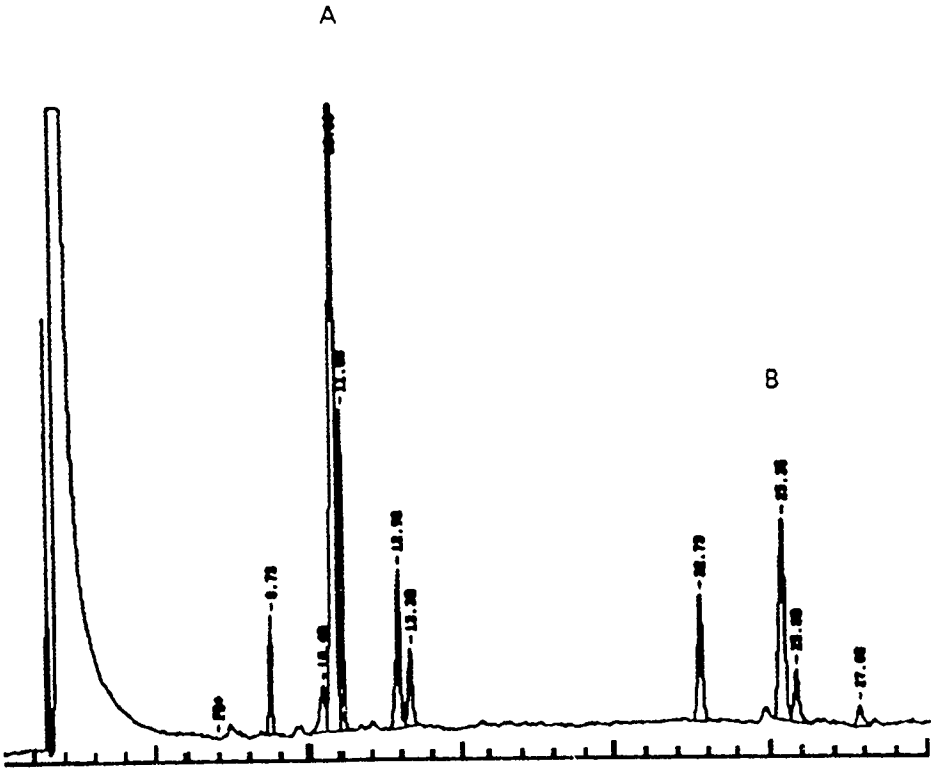

Figure 3.4-2. TCD Gas Chromatogram of CTFE .

EXPERIMENTAL APPROACH

Sampling

General Considerations: One-gram samples of tissue were initially placed in 20-mL scintillation vials. Urine and feces samples also were placed in scintillation vials for preparation. Tissue homogenizations and extractions of CTFE .nto hexane were performed in these vials Subsequent dilutions for analysis were accomplished by transferring aliquots of the extract to an appropriate volume of solvent in autosampler vials.

Blood

Rat blood samples were obtained by tail vein bleeding using an 80 µL capillary tube attached to a needle. Samples were handled quickly to reduce clotting or CTFE volatilization Blood was delivered slowly from the capillary tube into a 20-mL glass scintillation vial containing 5 0 mL of hexane The capillary was held below the hexane surface to reduce CTFE vaporization Hexane was pulled from the vial up through the capillary tube 10 times to wash remaining CTFE from the capillary wall into the sample. Scintillation vials with aluminum foil-lined caps were used once and discarded

to avoid contamination of subsequent samples. CTFE was extracted by mixing on an Evapotec® mixer for 2 h. Sample from the hexane layer was transferred to an autosampler vial.

Urine

Urine samples were collected daily from rats maintained in metabolism cages, and a sample was taken for analysis No preservative was used in order to avoid adding contaminants to the samples The total weight of daily urine collected was recorded Two hundred microliter samples of urine were pipetted into a 20-mL glass scintillation vial below the surface of the 5 0 mL-hexane. The urine was extracted for 1 h on an Evapotec® mixer Sample from the hexane layer was transferred to an autosampler vial.

Feces

Feces were collected daily from rats maintained in metabolism cages, and a sample was taken for analysis. No preservative was added to the samples The total weight of the feces collected was recorded A weighed sample of about 1 g was placed in a 20-mL glass scintillation vial containing 10 mL of hexane, mashed and then extracted The feces samples were extracted on an Evapotec® mixer overnight at room temperature Vials were centrifuged at 2000 x g for 10 min, the hexane layer was transferred to a 20-mL glass scintillation vial and stored at -70°C. Feces extracts were removed and allowed to thaw before transfer to an autosampler vial. These extracts were analyzed without dilution.

Tissue

Tissues were removed for extraction after separate sets of dissection instruments were used for skin opening and tissue removal The instruments were cleaned between tissues. The sequential surgical removal to reduce possible contamination of discrete organs was as follows. lungs, liver, right kidney, perirenal fat, right testicle, and brain Each tissue was weighed A sample of about 1 g was sectioned from each organ, weighed, and placed in a 20-mL glass scintillation vial containing 10 mL of hexane The vial was placed on ice until further processing. The tissue was homogenized in the vial using a Tissue-mizer then extracted overnight on an Evapotec® mixer at room temperature The vial was centrifuged at 2000 x g for 10 min, then the hexane layer was transferred to a 20-mL glass scintillation vial and stored at -70°C The extract vial was removed from the freezer and allowed to warm before handling Tissue extracts were diluted in an autosampler vial with hexane to reduce the CTFE in the most concentrated sample below 500 µg/mL for calibration purposes Lung, liver, testes, and brain extracts were diluted 1:20, kidney 1:50, and fat 1:1000

Gas Chromatographic Analysis and Data Handling

Conditions for the gas chromatographic analysis were identical to those used in the determination of distribution in the starting materials. The samples were processed using a Varian Autosampler, which permitted continuous analysis and enhanced the reproducibility of injections Data were collected using a Nelson Integration System that stored all sample data on disks for automated processing Raw data were processed on the Nelson System and transferred to an RS/1 program on a VAX computer system for final calculations of concentration

RESULTS

Test Material Analysis

CTFE oligomers were distributed into two discrete groups on the chromatograms The first peak in Group A had an 8.7-min retention time and comprised 4 area % of CTFE standards This peak was excluded because it was also found to be a possible metabolite in the urine and kidney extracts Group A then had five peaks with retention times ranging from 10 to 14 min Group B also had five peaks, with retention times ranging from 22 to 28 min.

Method calibration curves were developed from CTFE standard solutions in hexane An initial stock standard was prepared by pipetting hexane into a weighed amount of CTFE to obtain a stock standard solution. Standards over the working range of the method were prepared by serial dilutions of the stock solution. The usable standard range was from 1 to 500 µg/mL A best-fit polynomial was derived from concentration-response data obtained from standards. Concentrations were calculated for samples by comparison to the curve using an RS/1 program.

The best-fit polynomial was a quadratic equation. Several calibration curves were derived during the course of the study. Sample concentrations were calculated from the standard curve associated with each sample batch. A representative CTFE standard curve is shown in Figure 3 4-3

CTFE Concentration Summary Tables

Blood

CTFE concentrations for three 90-day exposure groups and two one-day exposure groups were determined at defined time intervals Each group contained four rats Blood extracts contained both sets of CTFE chromatographic peaks in roughly equal amounts No CTFE was found in the blood of control rats Figures 3.4-4 and 3 4-5 illustrate the changes in blood CTFE concentration with time The concentration of CTFE in blood drawn immediately after exposures showed that the 90-day exposure animals had concentrations twice that of the one-day exposed rats

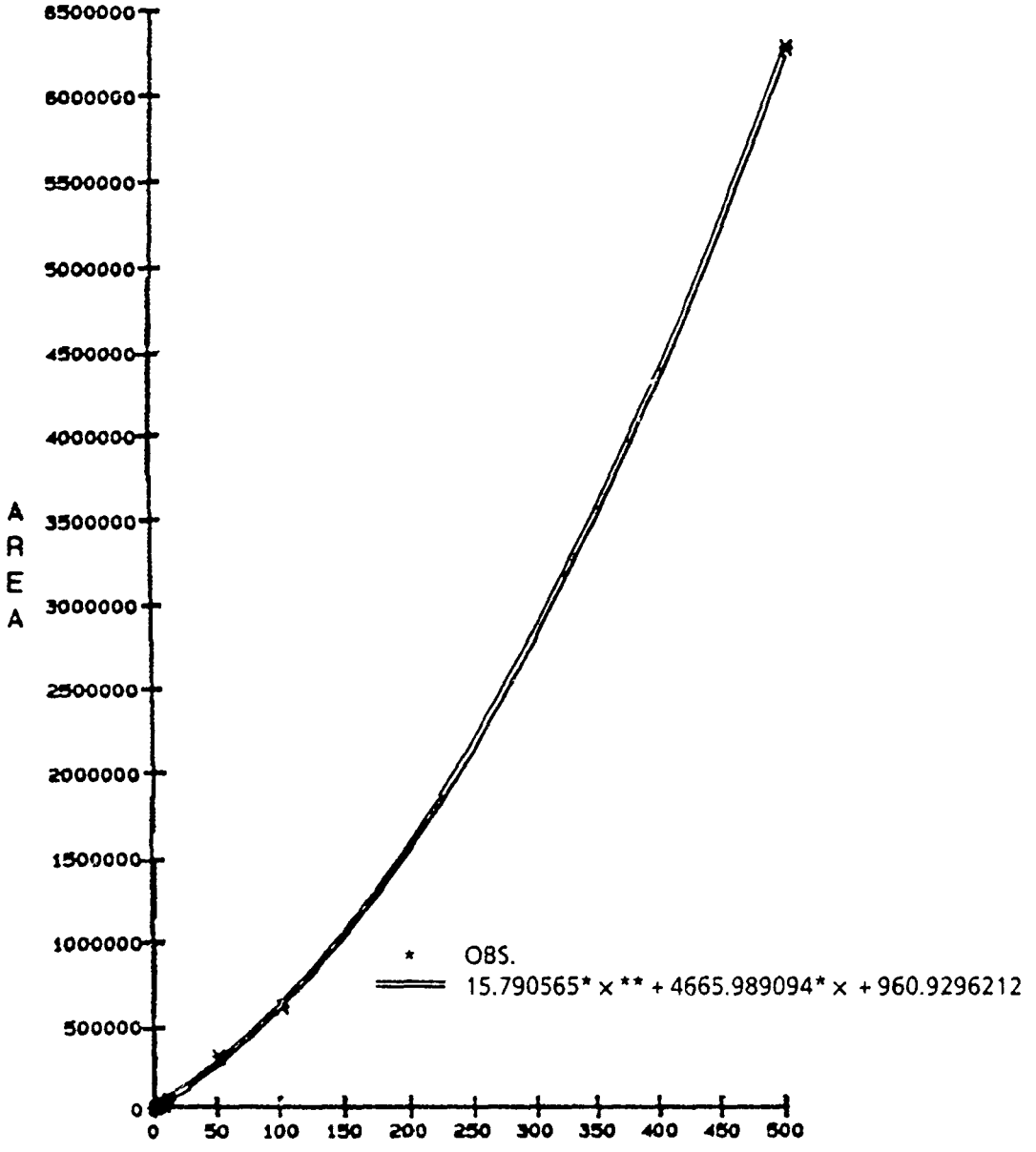

Figure 3.4-3. Typical CTFE Standard Curve

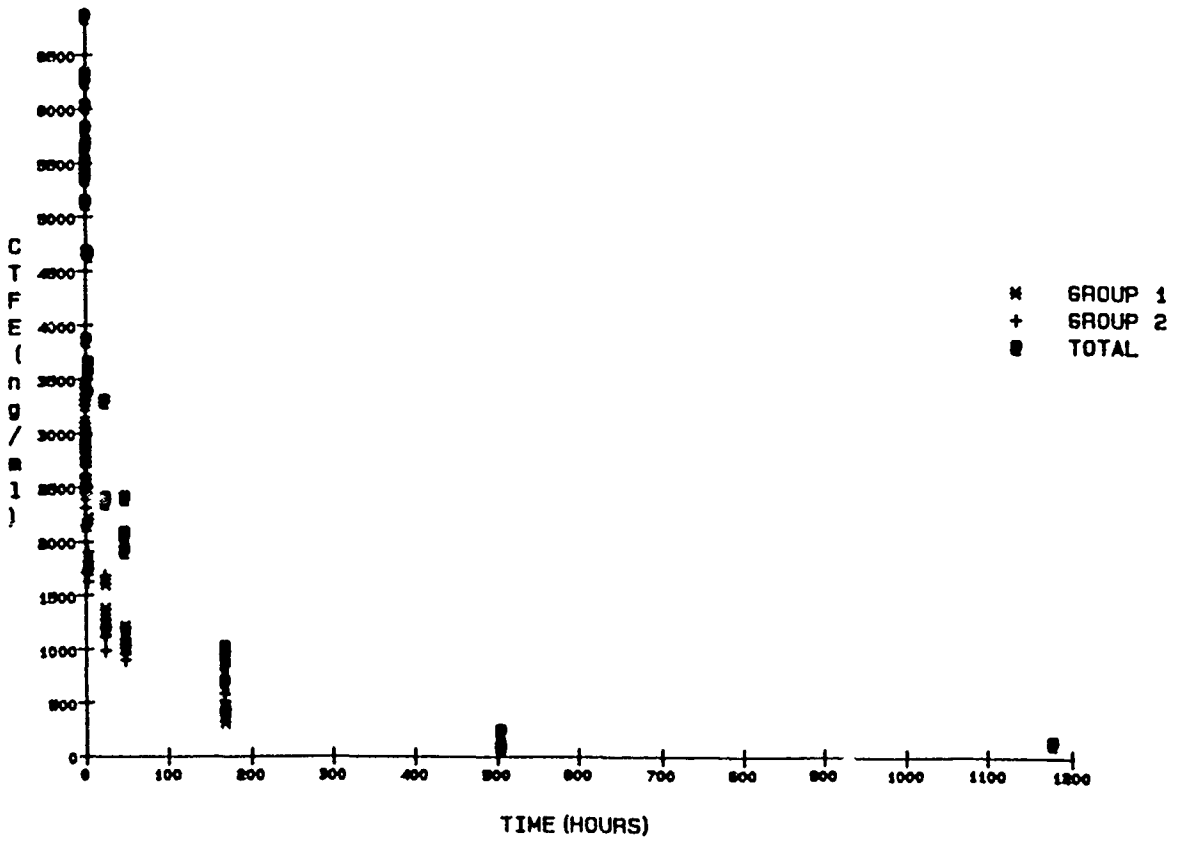

Figure 3.4-4. CTFE Blood Concentrations During a 90-Day Exposure.

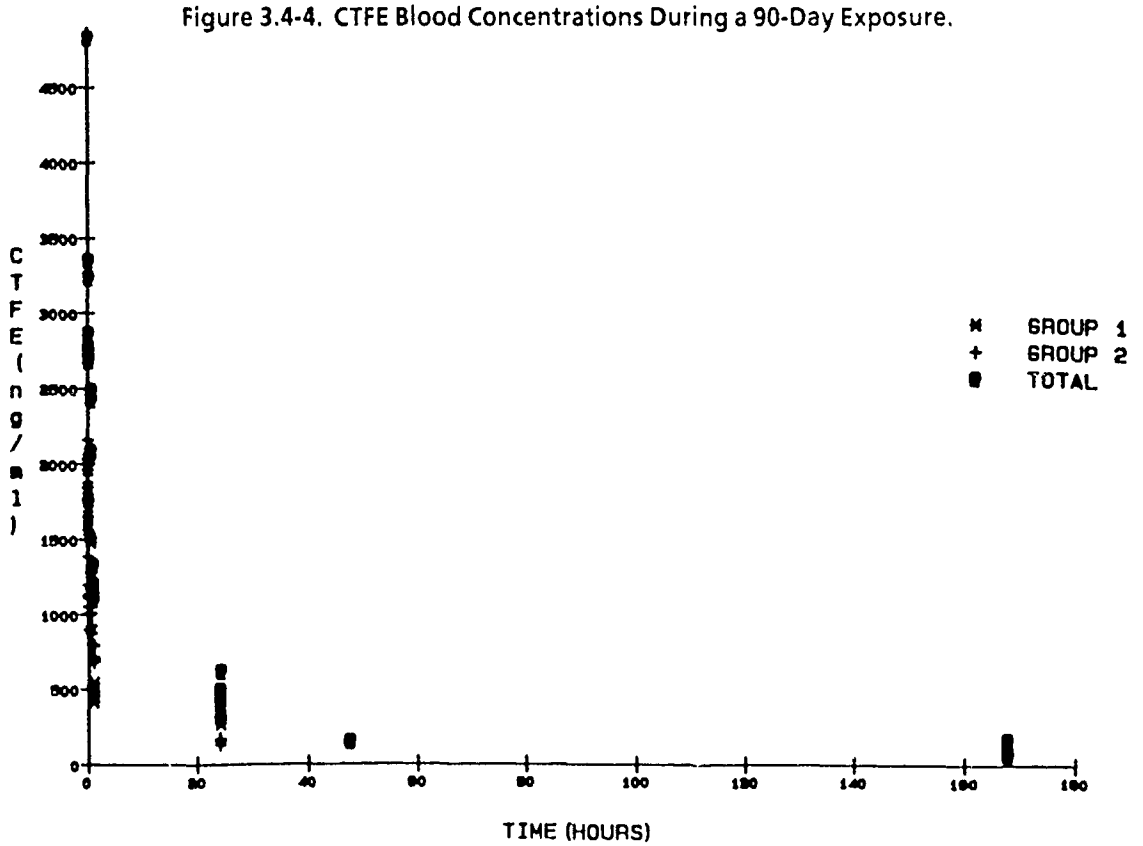

Figure 3.4-5. CTFE Blood Concentration Following 1 Day of Exposure.

Both 90- and 1-day CTFE concentrations initially decreased rapidly The one-day exposure rat blood concentration decreased much more rapidly than in the 90-day exposure animals After 48 h, there was a 13-fold difference in concentration between 1- and 90-day exposed animals CTFE concentration dropped below the detection limit after 500 h for the 90-day exposure sets and after 170 h for the one-day test rats.

Urine

Table 3 4-2 summarizes the concentrations of CTFE observed in urine excreted postexposure on a daily basis for 90- and 1-day exposure groups of four rats each. Only Group A CTFE peaks were extracted from urine. Control rats excreted no CTFE in urine. A graphic representation of these data versus time are shown in Figures 3.4-6 and 3.4-7 The total CTFE in urine was composed of chromatographic peaks from Group A. Urine from the 90-day test rats initially contained about nine times the CTFE concentration of the one-day test rats. CTFE was found in the 90-day exposure rat urine for 13 days postexposure. One-day test rats tested positive for CTFE for three days after exposure The 90-day rat urine excretion pattern was a general decreasing rate. The one-day test rats had an apparently random excretion pattern until the CTFE amount dropped below the detection limit. This later result is probably associated with the high degree of variability when measured CTFE levels are near the limit of detection.

TABLE 3.4-2. SUMMARY OF URINE CONCENTRATIONS OF CTFE

90-Day Test Animals			1-Day Test Animals		
Days Post	# of Animals	Avg. Conc. ngs	Days Post	# of Animals	Avg. Conc. ngs
1	4	1780	1	4	204
2	4	1965	2	4	107
3	4	1102	3	4	301
4	4	840	4	4	88
5	4	545	5	4	0
6	4	601	6	4	0
7	4	543	7	4	0
8	4	876	8	4	0
9	4	516	9	4	0
10	4	391			
11	4	326			
12	4	222			
13	4	182			
14	4	56			
21	4	77			
28	4	0			
35	4	0			
42	4	0			
49	4	0			

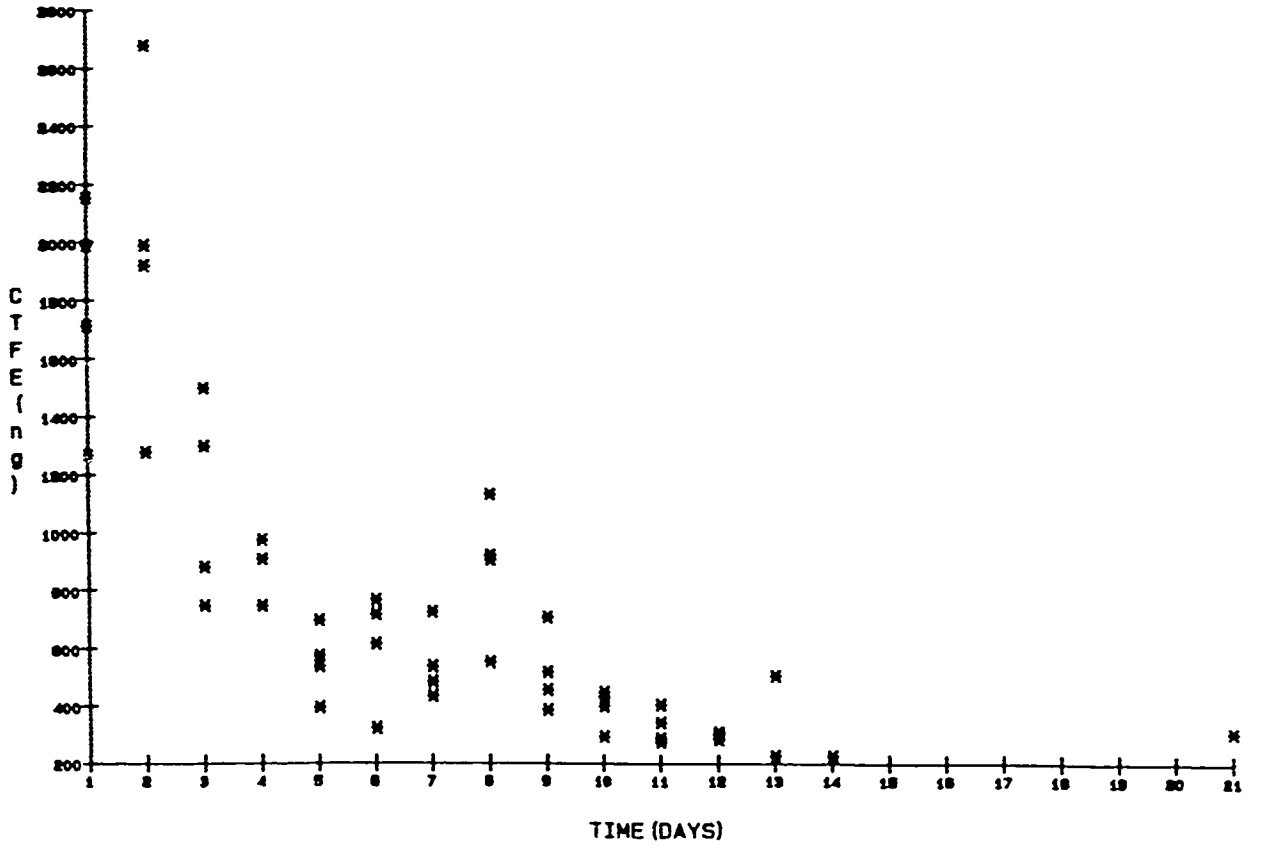

Figure 3.4-6. 90-Day Exposure CTFE Concentrations in Urine Over Time.

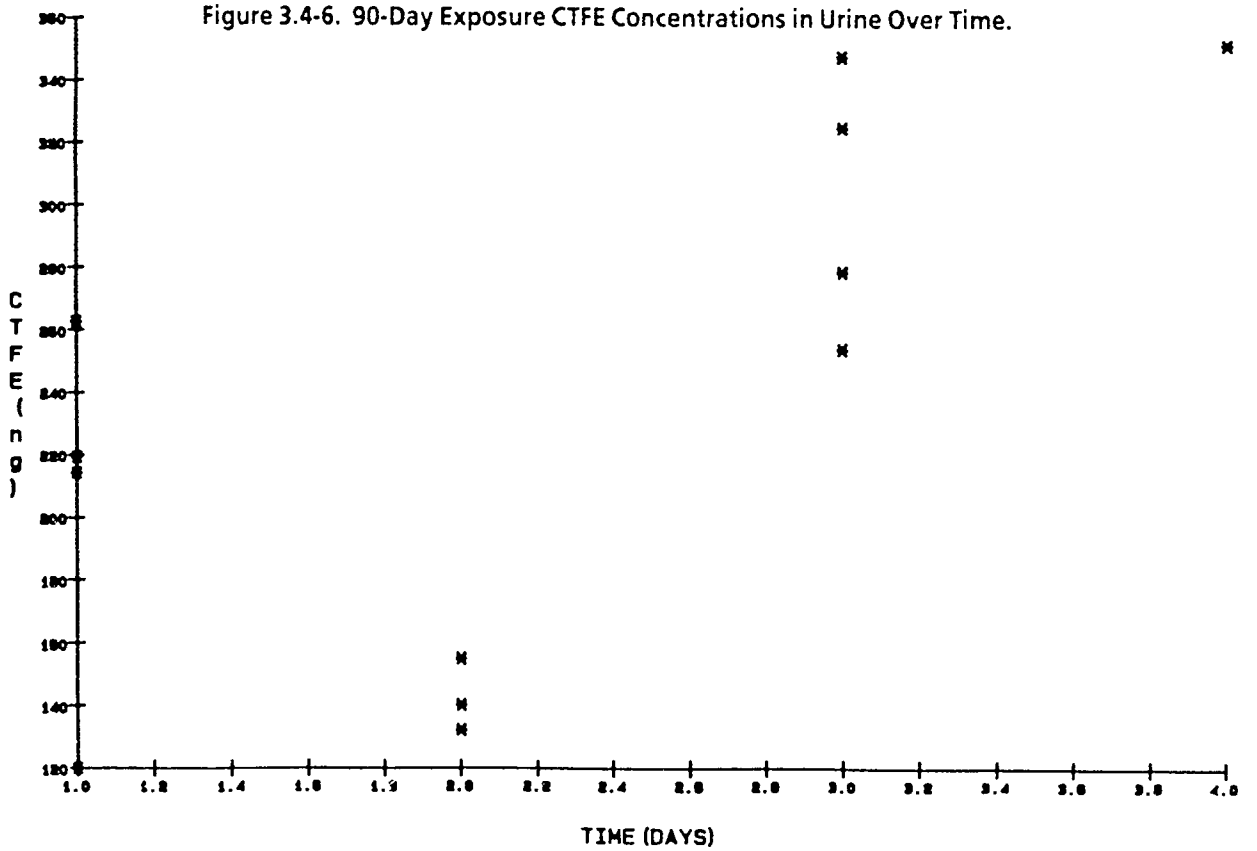

Figure 3.4-7. 1-Day Exposure CTFE Concentrations in Urine Over Time.

Feces

Chromatograms of feces extract of CTFE contained several small peaks not related to parent CTFE. The feces CTFE concentration was very low, the largest CTFE peak was the only peak detected.

Tissue

A summary of the concentrations of CTFE in tissue samples is shown in Table 3.4-3. Tissues were similar in CTFE concentration with the exception of fat. Fat tissue concentrations were about 40 times that of the other tissues. Comparison of rat tissue from 90-day exposed animals sacrificed immediately with tissue from rats sacrificed two days postexposure showed a 50% drop in CTFE tissue concentration except in fat which remained about the same. Group A chromatographic peaks decreased more rapidly than Group B peaks, possibly because these peaks were eliminated more readily through the kidney and the lung. The more rapid loss of Group A was seen also in tissue taken 145 days postexposure. At this time only trace amounts of CTFE remained as Group B oligomers and fat total CTFE concentration had decreased 94%.

TABLE 3.4-3. AVERAGE VALUES OF CTFE EXTRACTED FROM TISSUES (ngs CTFE/g TISSUE[a])

	Organ					
Animal Test Group	Kidney[b]	Lung[b]	Liver[b]	Testes[b]	Brain[b]	Fat[b]
Control	0	0	0	0	0	0
Control	0	378	0	0	0	0
1-Day Exposure Sac. 2 Days Post	3,245	8,136	1,176	1,093	1,304	47,117
1-Day Exposure Sac. 14 Days Post	977	2,308	0	180	0	43,893
90-Day Exposure Sac. 0 Days Post	60 441	51,336	48,503	22,977	25,432	1,614,457
90-Day Exposure Sacrifice 2 Days Post	32 196	30,011	24,139	11,864	12,486	1,728,560
90-Day Exposure Sacrifice 145 Days Post.	1,969	730	123	135	0	100,475

[a] These values are based on a theoretical extraction efficiency of 100% from tissue into hexane.
[b] Average values based on four animals per set except for the 145 day set in which only two animals were available.

Tissue samples were also taken from the one-day exposed rats. One set of animals was sacrificed two days after the one day exposure. A second set of one-day exposed animals was sacrificed 14 days after exposure. There was a wide variation in tissue CTFE concentrations from the one-day exposed rats sacrificed two days postexposure. Liver, testes, and brain were the tissues low in CTFE concentration. Kidney, lung, and fat CTFE concentrations were, respectively, 3, 6, and 40 times greater than the mean concentration of the lower tissue values. The decrease in CTFE concentration in the tissues can be seen by comparing the data from the one-day exposed rats sacrificed at two and

14 days postexposure Both Group A and B chromatographic peaks decreased in tissue, but Group A compounds decreased more rapidly

Control rat tissues contained no CTFE An interfering peak found in lung tissue among the Group B peaks was subtracted from the lung Group B and total concentrations A high percentage of the liver weight was blood that was lower in CTFE content For this reason, liver CTFE concentrations may have been biased low.

Possible Metabolites

Materials in addition to CTFE oligomers also were extracted Most prominent among these was a peak that co-eluted with the first CTFE peak. The retention times of this material and the CTFE peak were identical The first peak from Group A was not used to quantify CTFE oligomers in extracts because it was indistinguishable from this possible metabolite The large peak was present in urine and kidney extracts of exposed rats and not present in controls This peak was significantly larger than that of the corresponding CTFE oligomer The peak was tentatively identified as $C_5C1_5F_7$ by mass spectrometry.

Several small peaks were detected in various samples that were not present in control samples. However, these materials appeared to be random, with no consistent pattern.

DISCUSSION

Analyses of tissue, blood, and urine samples from animals exposed to CTFE have determined the distribution of the parent material. These data will be used to plan future exposure studies and to facilitate modeling of observed dose-response relationships

ACKNOWLEDGMENTS

Mr. Doug Helton and Ms. Joanne Drerup were responsible for the harvesting of tissues and the development of a surgical protocol to minimize cross contamination of organs. Larry Goodpaster, DVM, and Mr. Greg King prepared and extracted the tissue and excreta samples and obtained blood samples via tail vein bleeding Ms Kyung Yu provided mass spectral analysis and interpretation

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Kinkead, E.R., C.L. Gaworski, J.R. Horton, and T. R. Boosinger. 1987 Chlorotrifluoroethylene Oligomer. Evaluation of Acute Delayed Neurotoxicity in Hens and Study of Absorption and

Metabolism in Rats Following Oral, Dermal, and Inhalation Exposure AAMRL-TR-87, Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

3.5 INHALATION EXPOSURE TO CHLOROTRIFLUOROETHYLENE OLIGOMER MIXTURE – MEASUREMENT OF BONE AND URINE FLUORIDE

W.R. Sayers

INTRODUCTION

This report describes the analytical procedures used to determine fluoride in bone and urine as part of a 90-day inhalation toxicity study of chlorotrifluoroethylene (CTFE) It is one of a series of reports concerning CTFE exposure and toxicological evaluation

METHODS

Instrumentation

Fluoride measurements were performed with an Orion model 701A ionalyzer equipped with an Orion Research model 96-09-00 combination fluoride electrode The instrument was calibrated with standard fluoride solutions made from sodium fluoride Millivolt readings of standard solutions were plotted versus the base 10 logarithm of concentration to establish a calibration line for the instrument. A least-squares calculation was applied to the data to establish the slope and intercept for the line The regression line then was used to calculate fluoride concentration.

A Corning E-LX combination pH electrode was used for all pH measurements.

Materials

Reagent grade chemicals were used to prepare all buffers Standard stock solutions of sodium fluoride (Matheson SX/550CB721) were prepared by dissolving the appropriate amount of sodium fluoride in deionized water Calibration standards were prepared by serial dilution of the stock fluoride solution in deionized water (bone) or simulated urine (urine), using class A volumetric flasks

Total Ionic Strength Urinary Buffer

Total ionic strength urinary buffer was prepared by dissolving the salts listed in Table 3.5-1 in 500 mL of deionized water The buffer was then titrated to pH 5 25 with concentrated sodium hydroxide and finally diluted to 1 L The pH of the resulting buffer solution was approximately 5 2

TABLE 3.5-1. TOTAL IONIC STRENGTH BUFFER COMPOSITION

Compound	Amount
Acetic acid, glacial	57 mL
Sodium citrate	0 30 g
Sodium nitrate	57 80 g

Simulated Urine

Simulated urine was prepared by dissolving the materials listed in Table 3.5-2 in 1 L of deionized water.

TABLE 3.5-2. SIMULATED URINE COMPOSITION

Compound	Amount
Ammonium phosphate, dibasic	2.00 g
Sodium chloride	11.60 g
Sulfuric acid, concentrated	1.0 mL

Bone Analysis

A single femur from each animal was used for fluoride determination. Bones were cleaned by immersion in a 10% solution of papain (Walton, 1967) After the flesh was digested, the bones were rinsed with deionized water and dried overnight at 100°C in a vacuum oven. The clean, dry bones were broken up with a mortar and pestle and then ground to a fine powder using a ball mill Samples of 5 to 30 mg of powdered bone were weighed into nickel crucibles and ashed overnight in a muffle furnace. Ashing temperatures were from 550 to 750°C.

Fluoride levels in the bone ash were determined by the method of Singer and Armstrong (1968). Bone ash was quantitatively transferred to plastic beakers and dissolved in dilute hydrochloric acid. The ash solutions were then buffered and brought to a volume of 5 mL with deionized water Fluoride levels of the resulting solutions were determined using a fluoride electrode The results are reported as micrograms of fluoride per gram of bone

Urine Analysis

For urinary fluoride determinations animals were placed in metabolism cages and 24-h urine samples were collected and weighed The method used for urinary fluoride determination has been reported by Neefus et al (1970) Equal volumes of urine and total ionic strength urinary buffers were mixed After mixing, fluoride was determined using a fluoride electrode The results are reported as milligrams of fluoride excreted per 24 h

RESULTS AND DISCUSSION

Bone

A minimum of five bone fluoride determinations were made per animal The values given in Table 3.5-3 are the mean values for each animal

TABLE 3.5-3. BONE FLUORIDE[a]

Animal No.	Micrograms fluoride/ gram bone	Group Mean ± S.E.M.	Notes
Control Group[b]			
129	293	255 ± 53	
131	123		
132	274		
134	330		
Group 1[a]			
114	610	577 ± 56	Repeated exposure to 0.5 mg/L sacrificed 48 h postexposure
115	562		
117	682		
125	452		
Group 3[a]			
107	87	289 ± 147	Single exposure to 0.5 mg/L sacrificed 14 days postexposure
110	591		
113	408		
118	68		
Group 4[a]			
144	644	613 ± 91	Repeated exposure to 0.5 mg/L sacrificed immediately postexposure
155	819		
162	524		
166	463		
Group 6[a]			
136	298	326 ± 70	Single exposure to 0.5 mg/L sacrificed immediately postexposure
140	223		
143	282		
152	502		

[a] The data in Table 3.5-3 are presented graphically in Figure 3.5-1

As shown in Figure 3.5-1, the measured bone fluoride levels are highly variable. Data presented in the references also show a high degree of variability. This variability may due to the non-homogeneous nature of bones. The ends of the bones are the most biologically active and probably contain the most fluoride in exposed animals. The bone sampling procedure was not designed to control for intra-bone differences in fluoride deposition.

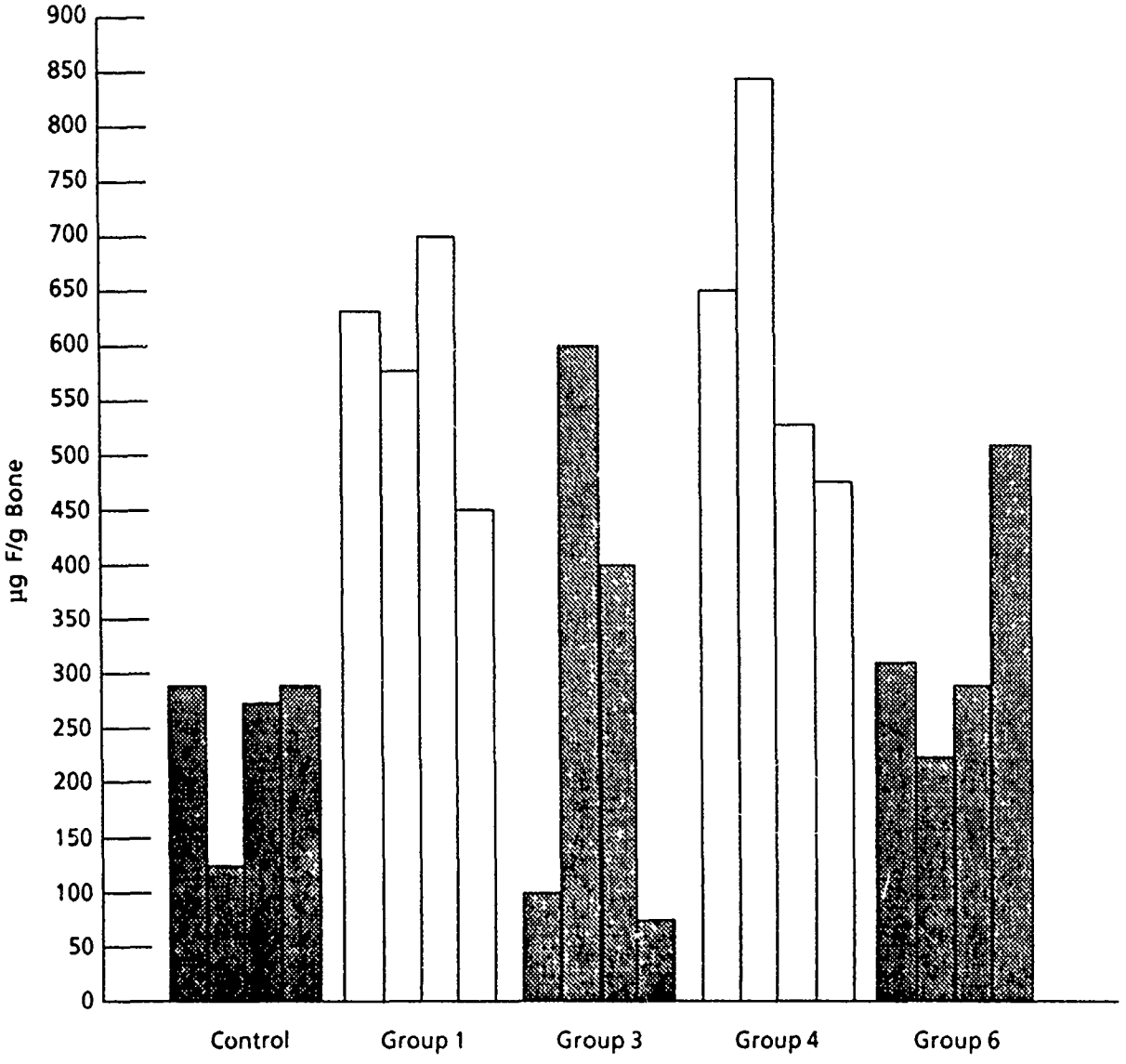

Figure 3.5-1. Bone Fluoride Concentrations.

Urine

The volume of each urine sample was calculated from urine weight using a value of 1 06 g/mL for the density of urine This value was determined experimentally for several urine samples. The fluoride concentration of each sample was then multiplied by the volume of the sample to give total fluoride excreted during a 24-h period The results in Table 3 5-4 are mean values for each group of animals expressed as µg of fluoride/24 h

TABLE 3.5-4. URINARY FLUORIDE[a]

Day of Study	Control[b]	Exposed for 90 days to 0.5 mg CTFE/L[b]
1	32	131
2	43	161
3	48	164
4	50	134
5	38	104
6	46	128
7	44	124
8	44	126
9	40	129
10	43	130
11	52	136
12	46	133
13	44	124
14	45	116
21	47	122
28	49	96
35	51	86
42	51	91
49	62	82
56	52	74
63	48	64
70	53	66
77	59	68
84	54	69
91	64	71

[a] A graphical representation of the results is shown in Figure 3.5-2
[b] µg Fluoride/24 h

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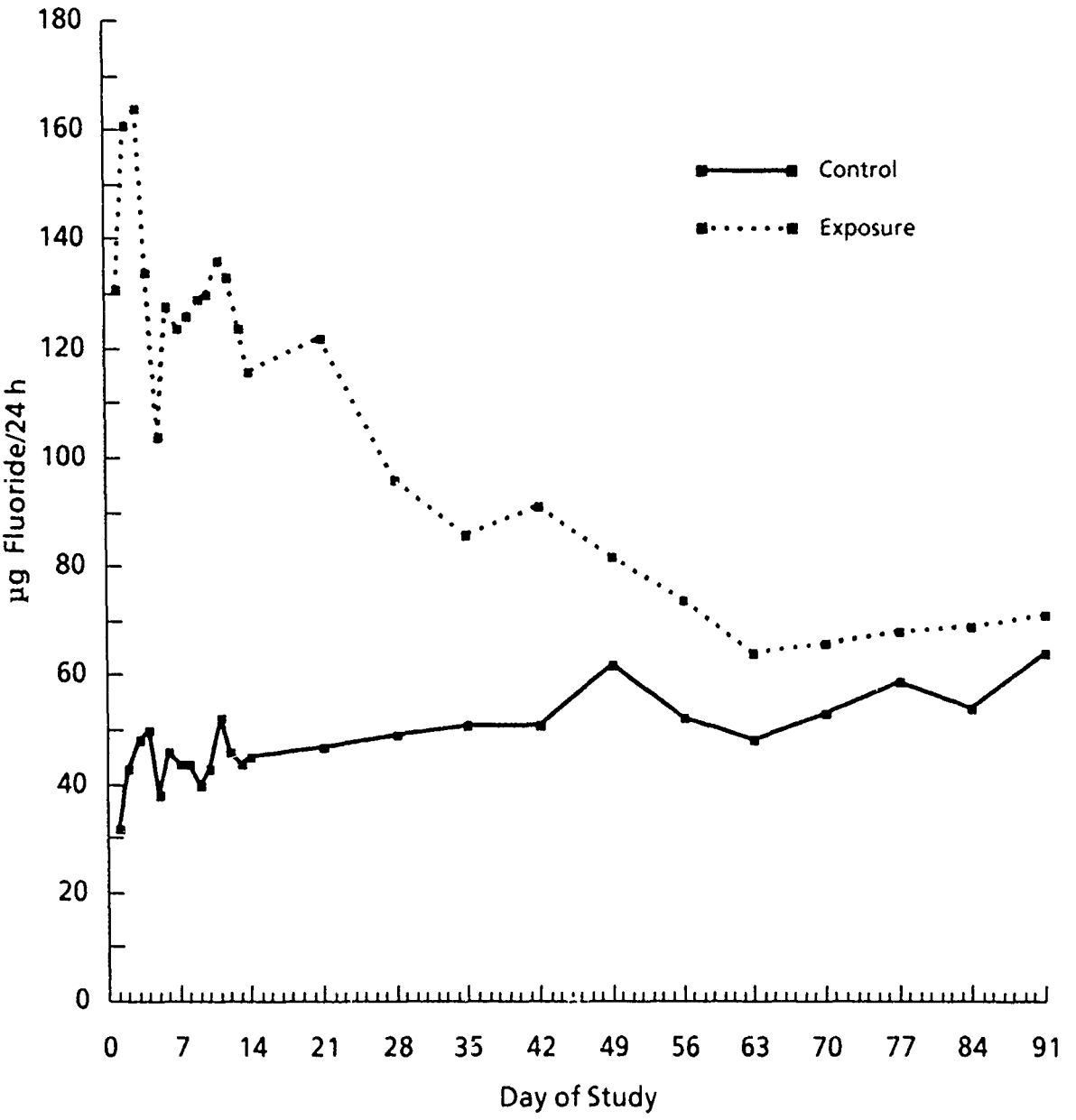

Figure 3.5-2. Urinary Fluoride Concentrations.

3.6 SUBCHRONIC STUDIES OF CHLOROTRIFLUOROETHYLENE

E.R. Kinkead, B.T. Culpepper, C.R. Doarn, E.C. Kimmel, H.G. Wall,
R.E. Whitmire[a], and D.R. Mattie[a]

INTRODUCTION

Chlorotrifluoroethylene (CTFE) oligomer is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectic strength Recent dermal and inhalation studies indicated that CTFE has a low degree of toxicity. There were no deaths among rabbits dermally exposed to 2 g CTFE/kg body weight (Gargus, 1983), and there were no deaths among rats exposed for 4 h to saturated-vapor concentrations of CTFE (Coate, 1984 and Kinkead et al , 1987).

Following oral and inhalation exposures, CTFE was readily absorbed and free fluoride was excreted in the urine Plasma and urine fluoride levels remained elevated for more than one week following oral exposure and for at least 24 h following inhalation exposure However, CTFE absorption was not evident following dermal exposure (Kinkead et al , 1987) Histopathologic examination of nerve tissue from hens dosed with CTFE showed no lesions of the type seen in organophosphate toxicity The acute toxicity evaluation of CTFE compares favorably with that of other hydraulic fluids tested in this laboratory

The subchronic inhalation tests reported here were conducted to provide information on health hazards likely to arise from repeated inhalation exposures over a limited time Data were collected to provide information on target organs from repeated inhalation exposures and for use in selecting dose levels for future chronic studies

METHODS AND EXPERIMENTAL EVALUATIONS

A detailed description of the methods and experimental evaluations performed for this study was provided in the 1987 Toxic Hazards Research Unit Annual Report (Kinkead et al , 1988)

RESULTS

Analyzed vapor-aerosol mixture concentrations were within 4% of the selected target values as listed in Table 3 6-1 The aerosol portion of the total chamber atmospheres averaged less than 10% at each concentration level No outward signs of toxic stress were observed and no exposure-related deaths occurred during the 90-day exposure period A male rat from the 0 50 mg/L group was euthanatized following accidental injury during the study A standard battery of serology assays performed on selected animals at the conclusion of the 90-day study was negative

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[a] AAMRL/TH Wright-Patterson Air Force Base OH

TABLE 3.6-1. ANALYSIS OF CTFE CONCENTRATIONS INHALED BY MALE AND FEMALE F-344 RATS FOR 90 DAYS

Target Concentration, mg/L	0 25	0.50	1.00
Mean Concentration, mg/L (N = 66)	0.25	0.48	0 98
Standard Error	0 002	0.003	0 004
Lowest Daily Average, mg/L	0.21	0 43	0.93
Highest Daily Average, mg/L	0.28	0.53	1.04
Percent Aerosol	5.30	6 80	8 90
MMAD	1 20	0 97	1 14
Geometric Standard Deviation	2 59	2 15	2 17

A significant difference in group mean body weights occurred at Day 0 of the study, which required that statistical analysis of all mean body weight data be performed on group mean body weight gains. With the exception of the 15- and 22-day weighings, the male control rats consistently gained body weight at a rate greater than any treated male rat group (Figure 3 6-1) The difference in body weight gains was greatest during the mid-portion of the study, Days 29 through 71, where a definite treatment-related effect occurred. Body weight gains in the treated female rats were slightly less than controls during the first three weeks of the study, but did not differ significantly thereafter.

Blood chemistry data obtained at sacrifice are listed in Tables 3 6-2 and 3 6-3 The mean alkaline phosphatase value for the male rats was significantly different ($p<0$ 01) from controls for all treatment groups with the high concentration group exhibiting a threefold increase over the control group Increased ($p<0$ 01) blood urea nitrogen (BUN) values were observed in the intermediate and high concentration male groups and increased serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were observed only in the high concentration male group. The treated female rats did not demonstrate differences from the control group

Notable, concentration-related, increases ($p<0$ 01) in relative kidney weights occurred in both sexes of rats (Tables 3 6-4 and 3 6-5) Relative kidney weights of the treated male rats were increased over controls by 30, 32, and 47% in the 0.25, 0 5, and 1 0 mg/L groups, respectively The female rats had relative kidney weight increases of 8, 11, and 19% in the respective treatment groups Similarly, a concentration-related increase in relative liver weights occurred in both sexes of rats Relative liver weights of the treated male rats were increased over controls by 84, 133, and 213% in the 0 25, 0 5, and 1 0 mg/L groups, respectively, and those of the treated female rats were increased over controls by 16, 34, and 77% at the respective exposure concentrations An increase in the relative testes weights of the treated male rats was noted; however, the absolute weights of the testes were comparable to those of controls

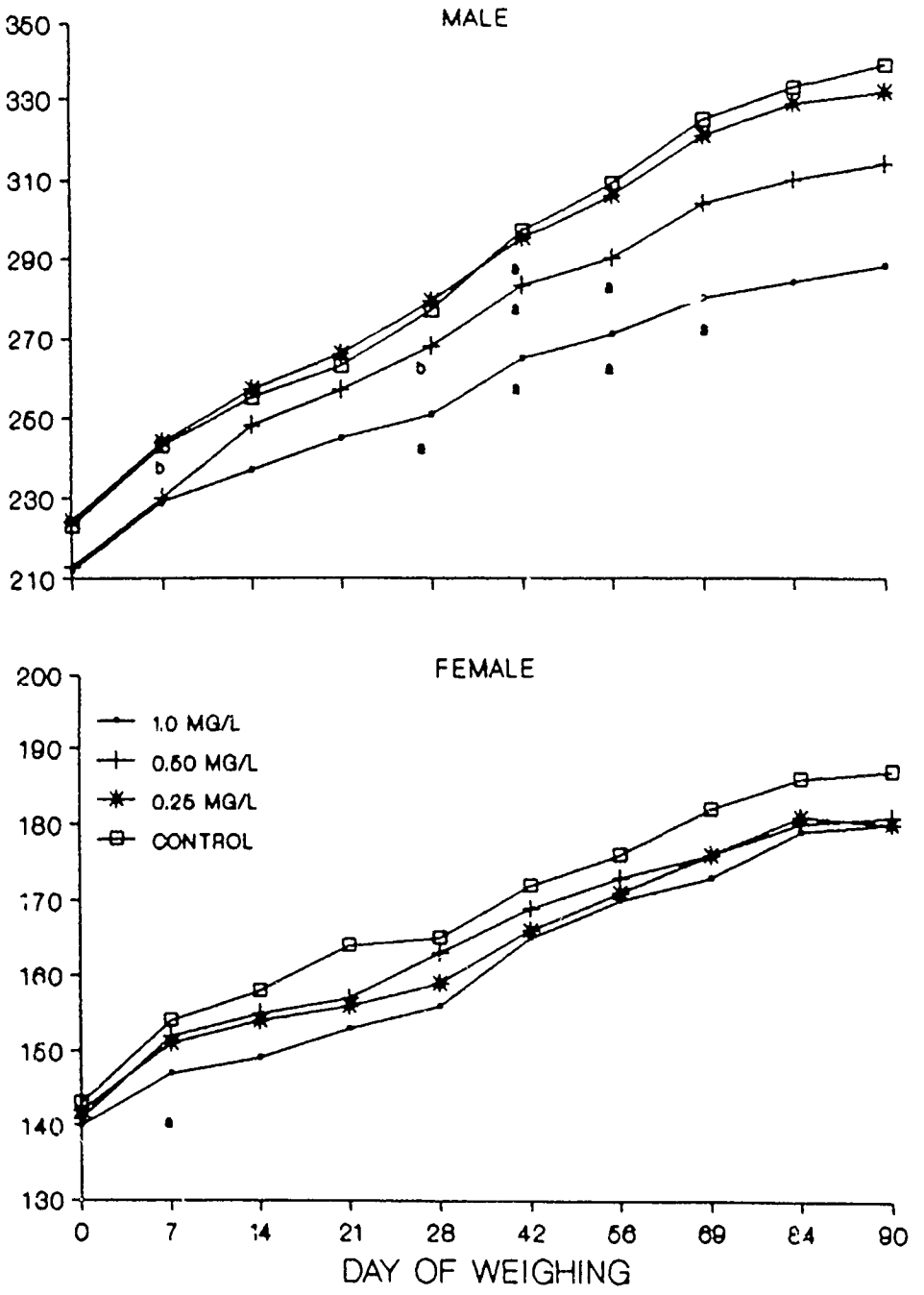

Figure 3.6-1. **Effect of 90-Day CTFE Inhalation Exposure on Body Weight Gain.** (N = 10 except for the 0.5 mg/L male group where N = 9 from Day 22 through 90. a = compared with control, $p < 0.01$; b = compared with control, $p < 0.05$ as determined by the Multivariate Analysis of Covariance for Repeated Measures Test.)

TABLE 3.6-2. BLOOD CHEMISTRY DATA[a] FROM MALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
BUN	11.7 ± 0 3(3)	14 1 ± 1 3(7)	17 4 ± 0 8(4)[b]	19 3 ± 1.2(6)[b]
CREATININE	0.60 ± 0 05 (5)	0 54 ± 0 05(5)	0 63 ± 0.05(4)	0 47 ± 0 09(6)
ALK PHOS	99.7 ± 3 2 (10)	148 1 ± 4 8(9)[b]	197 0 ± 6 6(6)[b]	345 1 ± 18 3(7)[b]
SGOT	73.7 ± 9 0 (10)	82 3 ± 8 2(9)	77 2 ± 6 9(6)	117 8 ± 6 8(6)[b]
SGPT	62.5 ± 4.4 (10)	78 4 ± 8 9(9)	68 7 ± 2 3(6)	112 7 ± 12 7(7)[b]

[a] Mean ± S E M
[b] Compared with control, p<0 01

TABLE 3.6-3. BLOOD CHEMISTRY DATA FROM FEMALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
BUN	14.3 ± 0.7(7)	14 6 ± 0 4(5)	13 0 ± 0 6(7)	14 5 ± 0.8(6)
CREATININE	0.51 ± 0.05 (7)	0 53 ± 0 07(3)	0 40 ± 0 1(3)	0 47 ± 0 04(6)
ALK PHOS	73.3 ± 2.2 (9)	91.0 ± 6 4(8)	89.4 ± 8 5(9)	89 1 ± 5.8(9)
SGOT	61.3 ± 8 1 (5)	68 8 ± 10 2(5)	68 7 ± 7 3(7)	52 5 ± 9.5(4)
SGPT	42.8 ± 6 3 (9)	53 7 ± 2 6(7)	43 6 ± 2.9(9)	47 6 ± 3 1(7)

TABLE 3.6-4. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
Kidney	2 48 ±0 06	3 14 ±0 09[c]	3 01 ±0 .04[c]	3 08 ±0 .04[c]
Ratio[b]	0.77 ±0 01	1 00 ±0 03[c]	1 01 ±0 01[c]	1 13 ±0 01[c]
Heart	1.12 ±0 03	1 07 ±0 02	1 02 ±0 03[d]	0 98 ±0 02[d]
Ratio	0.35 ±0 00	0 34 ±0 01	0 34 ±0 01	0 36 ±0 01
Brain	1 91 ±0 01	2 00 ±0 01	1 95 ±0 02	1 92 ±0 02
Ratio	0 60 ±0 01	0 62 ±0 01	0 66 ±0 00	0 70 ±0 01
Liver	9 12 ±0 22	16 46 ±0 26[c]	19 65 ±0 40[c]	24 27 ±0 43[c]
Ratio	2.83 ±0 04	5 22 ±0 07[c]	6 61 ±0 11[c]	8 87 ±0 11[c]
Spleen	0 67 ±0 01	0 67 ±0 01	0 67 ±0 02	0 62 ±0 02[d]
Ratio	0 21 ±0 00	0 21 ±0 00	0 23 ±0 00	0 23 ±0 01
Thymus	0 39 ±0 15	0 24 ±0 01	0 24 ±0 01	0 22 ±0 02
Ratio	0 12 ±0 05	0 08 ±0 00	0 08 ±0 00	0 08 ±0 01
Lungs	2.04 ±0 06	2 00 ±0 04	2 21 ±0 35	1 89 ±0 03
Ratio	0.64 ±0 02	0 63 ±0 01	0 74 ±0 01	0 69 ±0 01
Adrenal	0.07 ±0 .00	0 08 ±0 01	0 08 ±0 00	0 09 ±0 00
Ratio	0 02 ±0 00	0 02 ±0 00	0 03 ±0 00	0 03 ±0 00
Testes	3 24 ±0 03	3 31 ±0 04	3 28 ±0 04	3 21 ±0 04
Ratio	1 01 ±0 02	1 05 ±0 01[d]	1 10 ±0 01[c]	1 18 ±0 02[c]
Whole Body	322 2 ± 6 0	315 5 ± 3 5	297 4 ± 3 7[c]	273 7 ± 4 5[c]

[a] Mean ± S E M , N = 10 for all groups except the 0 50 mg L group where N = 9
[b] Organ weight/body weight x 100
[c] Compared with control, p<0 01
[d] Compared with control, p<0 05

TABLE 3.6-5. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
Kidney	1 45 ± 0 02	1 52 ± 0 03	1 57 ± 0 02	1 66 ± 0 02[d]
Ratio[b]	0 81 ± 0 01	0 89 ± 0 01[c]	0 90 ± 0 01[c]	0 97 ± 0 01[c]
Heart	0 70 ± 0 02	0 71 ± 0 02	0 71 ± 0 02	0 73 ± 0 02
Ratio	0 39 ± 0 01	0 41 ± 0 01	0 41 ± 0 01	0 42 ± 0 01
Brain	1 78 ± 0 02	1 79 ± 0 02	1 77 ± 0 03	1 81 ± 0 01
Ratio	1 00 ± 0 02	1 03 ± 0 02	1 02 ± 0 01	1 05 ± 0 01
Liver	4 71 ± 0 07	5 30 ± 0 08	6 12 ± 0 07[c]	8 01 ± 0 07[c]
Ratio	2 64 ± 0 04	3 06 ± 0 05[c]	3 52 ± 0 04[c]	4 67 ± 0 06[c]
Spleen	0 44 ± 0 01	0.47 ± 0 01	0 48 ± 0 01	0 49 ± 0 01
Ratio	0 25 ± 0 01	0 27 ± 0 01	0 28 ± 0 01	0.29 ± 0 01
Thymus	0.20 ± 0 01	0 18 ± 0 01	0 20 ± 0 .01	0.21 ± 0 .01
Ratio	0 11 ± 0 01	0 11 ± 0 01	0.12 ± 0 01	0.12 ± 0 01
Lungs	1.43 ± 0 04	1 49 ± 0 09	1 34 ± 0 05	1.58 ± 0 .06
Ratio	0.80 ± 0 02	0 84 ± 0 05	0 77 ± 0 02	0 92 ± 0 03
Adrenal	0 08 ± 0 00	0 09 ± 0 00	0 09 ± 0 00	0.09 ± 0 00
Ratio	0.04 ± 0 .00	0 05 ± 0 00	0.05 ± 0 00	0.05 ± 0 00
Ovary	0.14 ± 0 01	0.15 ± 0 01	0 15 ± 0 01	0 14 ± 0 01
Ratio	0 08 ± 0 00	0 09 ± 0 01	0 09 ± 0 01	0 08 ± 0 00
Whole Body	178 8 ± 2 5	173.4 ± 2 9	173 7 ± 1 9	171 9 ± 2 2

[a] Mean ± S E M , N = 10 for all groups except the 0 50 mg/L group where N = 9
[b] Organ weight/body weight x 100
[c] Compared with control, p<0 01
[d] Compared with control, p<0 05

Gross pathologic findings at the conclusion of the 90-day exposures consisted of gross liver enlargement in all CTFE-exposed rats that was subsequently determined to be statistically significant when compared to controls (p<0 01) Among female rats, the incidences of grossly detected paraovarian cysts were 40, 10, 10, and 0% in the control, 0 25, 0 5, and 1 0 mg CTFE/L dose groups, respectively

Histologically, the incidence of hepatocytomegaly was 100% in CTFE-exposed male and female rats at each test concentration with no occurrence of this lesion noted in control rats of either sex The level of significance of hepatocytomegaly in each CTFE-exposed group of rats was p<0 01 (Table 3 6-6) Among male rats exposed to 1 0 and 0 5 mg CTFE/L, 9 of 10 and 8 of 10 rats, respectively, had hyaline droplet accumulation in the kidney proximal tubules Compared to the control male rats, the incidence of hyaline droplet accumulation for each test group was statistically

different at the $p<0.01$ level The histopathologic examination confirmed each gross finding of paraovarian cysts in female rats and also revealed paraovarian cysts that had not been grossly detected in other female rats The microscopic incidences of paraovarian cysts were 6 of 10, 7 of 10, 9 of 10, and 9 of 10 among the control, 0.25, 0 5, and 1 0 mg CTFE/L groups No statistical differences between groups were noted for the incidence of this lesion

TABLE 3.6-6. INCIDENCE (%) SUMMARY OF SELECTED MICROSCOPIC LESIONS OF RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Male				Female			
	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
Liver								
Hepatocellular Cytomegaly	0	100[a]	100[a]	100[a]	0	100[a]	100[a]	100[a]
Kidneys								
Laminated Concretions	100	100	100	90	100	90	90	100
Hyaline Droplet Formation	0	0	80[a]	90[a]	0	0	0	0
Ovaries								
Paraovarian Cyst	–	–	–	–	60	70	90	90

[a] Compared with control, $p<0.01$

Additional histopathologic findings, with treatment/sex group incidences of two or fewer rats affected, included colonic nematodiasis, focal myocarditis, renal retention cysts, pulmonary subpleural histocytosis, focal dacroadenitis, rhinitis, and multifocal chronic hepatitis One male rat in the high concentration group had a nephroblastoma in one kidney Ninety percent or greater of the 10 rats in each of the male and female control and CTFE-exposed groups had a few foci of laminar concretions within renal tubules

Descriptively, the light microscopic liver lesions consisted of multifocal to diffuse enlargement of individual hepatocytes with a massive increase in the cytoplasm and slightly increased nuclear size Compared to unaffected cells in controls, individual hepatocytes in CTFE-exposed rats had at least a twofold increase in size. The cytoplasm exhibited a loss of the normal basophilic stippling and was replaced by eosinophilic granularity The engorged cells distorted the hepatic cords and, in some areas, obliterated sinusoids. The liver lesions in male rats were more severe than in female rats at each CTFE concentration level The liver lesions in male rats were more diffusely distributed than in female rats Females had multifocal centrolobular-oriented lesions Attempts to grade the lesions according to CTFE concentration level proved unsuccessful as differences in the severity of lesions between the 1.0 and 0.5 mg CTFE/L groups were morphologically indistinguishable

Table 3 6-7 presents data from the morphometric examination of hepatocytic ultrastructure in livers from control and CTFE-exposed rats The ultrastructural examination revealed mild to moderate mitochondrial swelling of rats of both sexes exposed to CTFE In CTFE-exposed rats, hypertrophied hepatocytes had increased amounts of smooth endoplasmic reticulum (SER) and peroxisomes The relative amounts of SER were increased in a concentration-dependent manner in hepatocytes from both sexes, with no difference between sexes The number of peroxisomes per visual field also was increased in a concentration-dependent manner The peroxisomes were significantly increased in male rat hepatocytes as compared to female rat hepatocytes Rough endoplasmic reticulum (RER) was unaffected in female CTFE-exposed rats, while male CTFE-exposed rats had less RER as the amount of SER increased Lipid vacuoles were increased in male rats after CTFE exposure, but decreased in female rats after CTFE exposure The number of lipid vacuoles present was not treatment-dependent In CTFE-exposed rats, membranous profiles were seen in the cytoplasm of male rat livers at each concentration level, but were seen only in hepatocytes of female rats exposed to 0.5 and 1 0 mg CTFE/L

TABLE 3.6-7. MORPHOMETRIC ULTRASTRUCTURAL HEPATOCYTE EVALUATION OF RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

		CTFE Exposure[a] Groups[b,c]			
Sex	Organelle	0.0	0.25	0.50	1.0
Males	Mitochondria	0 0 ± 0 0	1 3 ± 0 6	1 2 ± 0 7	2 1 ± 0 62
	SER	1 0 ± 0 6	1 7 ± 0 3	2 0 ± 0 0	3 3 ± 0 3
	Peroxisomes	3 2 ± 0 6	7 7 ± 2 0	16 3 ± 3.1[d]	15 2 ± 0 0[d]
Females	Mitochondria	1.2 ± 0 2	1 7 ± 0 3	1.8 ± 0.3	2 3 ± 0 4
	SER	1 0 ± 0 0	1 3 ± 0 3	2 4 ± 0 3	3 0 ± 0 0
	Peroxisomes	4.4 ± 0 4	8 1 ± 0 6	7 2 ± 0 6[e]	8 2 ± 0 9[e]

[a] CTFE exposure concentration expressed as mg/L
[b] Group size, N = 3
[c] Mean ± S E M for grades of severity of mitochondrial swelling and relative amounts of smooth endoplasmic reticulum and peroxisome proliferation
[d] Significantly different from control and 0 25 mg/L groups at $p < 0.05$
[e] Significantly different from corresponding male rats at $p < 0.05$

The kidney lesions were limited to the epithelial cells of the proximal convoluted tubules and consisted of hyaline droplet accumulation within the cytoplasm and laminar concretions that appeared to be anchored to the basement membrane The second lesion, laminar concretions, was present in most rats of either sex among the control and CTFE-exposed groups The hyaline droplet formation appeared to be slightly more severe in male rats in the high concentration (1 0 mg CTFE/L) group, in that the number of affected proximal convoluted tubule epithelial cells and the number and size of droplets appeared to be greater in this group

Two of four rats included in this study for pharmacokinetic modeling and exposed to 0 5-mg CTFE/L died, while anesthetized, prior to the beginning of the surgical procedure to collect a liver specimen at 105 days postexposure The light microscopic examination did not reveal pathologic alterations in the liver of control animals Histopathologic examination of liver specimens from the two rats that died and two CTFE-exposed rats that survived the biopsy procedure disclosed diffuse hepatocytomegaly with cytoplasmic eosinophilic granules and variable-sized clear vacuoles. Via light microscopy, the vacuolar changes were suggestive of lipid accumulation. Multifocal aggregates or single enlarged hepatocytes with poorly stained ground-glass cytoplasm and no apparent nuclear alteration were seen also in livers of the CTFE-exposed rats Via periodic acid-Schiff staining, glycogen could not be detected in hepatocytes possessing ground-glass cytoplasm, but was present in other non-necrotic hepatocytes Foci of hepatocytic necrosis with and without associated inflammation were seen in liver sections of CTFE-exposed rats The foci of hepatic necrosis were usually associated with enlarged hepatocytes with extensive cytoplasmic vacuolation or poorly stained cytoplasm

At 236 days after cessation of inhalation exposure to 0 5 mg CTFE/L, the hepatocytes of the CTFE-exposed rats still possessed an increased amount of eosinophilic cytoplasmic granularity, compared to controls, and occasionally contained microvacuoles suggestive of fat accumulation. The hepatocytes were essentially the same size as most hepatocytes in control rats, differing from the hepatocytomegaly seen in livers of CTFE-exposed rats immediately postexposure and at 105 days postexposure Compared to hepatocytes in livers of CTFE-exposed rats at earlier postexposure points, the cytoplasm in hepatocytes of CTFE-exposed rats contained more basophilic material at 236 days after CTFE exposure The increased basophilia may be attributable to increased abundance of RER or reduced proliferation of organelles that masked the demonstration of basophilic organelles at earlier postexposure points. Both control and CTFE-exposed rats had chronic pericholangitis with biliary duplication and foci of hepatocytic necrosis These foci were probably due to post-surgical portosystemic vascular shunts, biliary leakage, and bile duct recanalization following the 105-day postexposure biopsies

DISCUSSION

Repeated 90-day inhalation of CTFE vapors resulted in depression of body weight gains throughout the exposure period and appeared to be treatment-related, particularly in the male rat groups A transient depression of mean body weight gains was noted in female rats, but only during the first two-week period and only in the highest concentration tested

The changes in alkaline phosphatase, SGOT and SGPT, were considered directly related to CTFE exposure since there was significant morphologic alteration in hepatocytes of all CTFE-exposed rats The hypertrophy of hepatocytes with resultant compression of sinusoids suggested that there was

concurrent compression of the biliary duct system These effects could have contributed to intrahepatic biliary obstruction and the subsequent induction of alkaline phosphatase synthesis and release to blood. SGPT and SGOT elevations were probably a consequence of hepatocytic necrosis, a feature seen most frequently in the high concentration male rats BUN mean values, though statistically significantly different from the control group mean, were not considered to have pathophysiologic significance because they are within the 12 6 to 35 8 mg/dL range reported by the clinical pathology laboratory for historical controls Although hyaline droplet accumulation in proximal tubules was prevalent in the median- and high-dose male rat kidneys, renal epithelial necrosis or other morphologic indicators of significant renal disease were not present

Of prime importance was CTFE's toxic effect on the liver Many of the hepatotoxic effects of CTFE appeared to be treatment- and sex-dependent The morphologic results document gross liver hypertrophy and microscopic hepatocytomegaly as the principal manifestations of CTFE-induced hepatotoxicity The electron microscopic examination demonstrated peroxisomal proliferation and increased smooth endoplasmic reticulum as the primary structural factors responsible for the hepatocytomegaly Treatment-dependent morphologic changes in CTFE-exposed rats included increased SER in hepatocytes of both sexes of rats, increased cytoplasmic membranous profiles in hepatocytes of female rats exposed to median and high concentrations of CTFE, and progressively decreased amounts of RER in hepatocytes of male CTFE-exposed rats as the CTFE concentration increased The sex-dependent hepatotoxic effects demonstrated via light microscopy were diffuse hepatocytomegaly in male CTFE-exposed rats and multifocal centrolobular-oriented hepatocytomegaly in female rats

Increased hyaline droplet formation is usually limited to male rats and tend to increase spontaneously in severity with age Several hydrocarbon fuels are known to induce hyaline droplet formation (Bruner, 1984) The organophosphate dimethyl methylphosphonate also induces renal tubular hyaline droplet formation (Mattie and Hixson, 1987) Usually accompanying renal tubular necrosis has been observed when hyaline droplet formation has been considered to have pathophysiologic significance Renal tubular necrosis was not a feature in CTFE-exposed rats, therefore, hyaline droplet formation in male rat kidneys after CTFE exposure may not be a toxicologically significant effect

The finding of a nephroblastoma was probably unrelated to CTFE toxicity despite its occurrence in a rat that received a 1 0 mg CTFE/L exposure The tumor occasionally has been found in rats and is most commonly found in younger rats having a mean age less than one year (Altman and Goodman, 1979) Other gross and histologic findings reported in the results were also background lesions that probably had little interaction with the toxic effects of CTFE

Following a 90-day inhalation exposure to CTFE, the hepatocytic injury apparently became more severe as evidenced by multifocal enlargement of cells that became necrotic by 105 days postexposure. Although abnormal hepatocytic eosinophilic granularity persisted, hepatocytes from CTFE-exposed rats appeared to be partially recovered at 236 days postexposure, as indicated by reduced cytoplasmic volume and reduction in the frequency and severity of cytoplasmic vacuolar degeneration

A retrospective histopathologic review of archived rat liver histology slides from a subchronic study of the effects of orally administered Halocarbon 27-S, another mixture of CTFE oligomer, revealed slightly increased hepatocytic volume, accumulation of eosinophilic cytoplasmic granules, and reduced cytoplasmic basophilia Perfluorodecanoic acid (PFDA), a chemical that is structurally unrelated to CTFE, also causes hepatocytic enlargement, though less than that caused by CTFE in this study Light microscopically, the enlarged hepatocytes have increased cytoplasmic eosinophilic granularity with reduced cytoplasmic basophilia Electron microscopic studies of hepatocytic effects caused by PFDA exposure demonstrated peroxisome proliferation and cytoplasmic accumulation of membranous profiles. Several hypolipidemic pharmaceuticals and industrial plasticizers are also known to cause peroxisome proliferation in hepatocytes (Reddy and Lalwani, 1983).

The biochemical mechanisms that account for the toxicologic effects of CTFE oligomer are unclear presently Speculatively, the peroxisomal proliferation due to CTFE may have a central role in hepatotoxicity leading to dead hepatocytes Although hepatocytes apparently begin to recover, the presence of membranous profiles suggests that oxidative cell injury occurs This effect may be due to increased peroxide generation within hepatocytes having increased peroxisomes Though unproven, sex-related metabolic differences probably account for the increased hepatotoxicity of CTFE in male rats as compared to female rats

On the basis of results from studies of six hypolipidemic compounds, the hypothesis has been advanced that chemicals that are potent peroxisome proliferators are carcinogenic (Reddy et al , 1980) Peroxisome proliferation is proposed to be an endogenous indirect initiator of carcinogenesis. As a consequence of peroxisomal β-oxidation of fatty acids, hydrogen peroxide and its reduction products accumulate in hepatocytes The concentration of intracellular peroxides formed by fatty acid oxidation is believed to be too low to be destroyed by peroxisomal catalase, yet sufficient to cause damage to DNA, lipid peroxidation, or oncogene activation when the number of hydrogen peroxide generating peroxisomes is increased (Reddy and Lalwani, 1985)

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3.7 INHALATION EXPOSURE TO CHLOROTRIFLUOROETHYLENE OLIGOMER: *IN VIVO* ESTIMATION OF PARTITION COEFFICIENTS

D.L. Pollard and R.L. Carpenter

ABSTRACT

Measurements of both blood and organ chlorotrifluoroethylene (CTFE) concentrations allowed calculation of estimated blood/organ partition coefficients from data obtained during an inhalation toxicity study. This paper describes the method used and presents results of these calculations.

INTRODUCTION

The CTFE 3.1 oil used in this study is a nonflammable, saturated, halocarbon oil containing no hydrogen. It is an example of a class of oils and lubricants having differing molecular weights produced by polymerizing CTFE and stopping the reaction by adding chlorine to the growing polymers. This report is one of a series describing a 90-day inhalation exposure of rats to CTFE. In addition to testing the inhalation toxicity of CTFE, the exposure study was intended to gather data on the time-dependent tissue concentrations of CTFE. These data were used as a basis for developing a physiologically based pharmacokinetic model for simulation of CTFE uptake, distribution, and elimination. An important physical parameter of such a model is CTFE tissue solubility in blood and organ tissues of interest. Normally, the relative solubility of the test material for such a study would be measured in the form of a partition coefficient obtained from *in vitro* experiments, although *in vivo* methods have been reported (Lutz et al.,1977 and Lin et al., 1982). During the CTFE study, a group of rats were exposed long enough that CTFE in their internal organs approached equilibrium with their blood. Measurements were made of the CTFE concentrations in both blood and selected tissues. Since the animal closely approached equilibrium, the ratio of these concentrations can be used as an estimate of the solubility of CTFE in the respective tissues. These assumptions are valid only to the extent that metabolic activities within the live animal do not alter the thermodynamically determined equilibrium Thus, this approximation would not be valid in the face of extensive CTFE metabolism within an organ or active transport of the CTFE.

MATERIALS AND METHODS

Rats were removed from the exposure chambers and immediately tail bled using a capillary tube on a syringe The blood sample obtained was injected into hexane and mixed to extract CTFE. Care was taken not to expose the blood to air to avoid CTFE loss. The rats were immediately sacrificed, and tissues were harvested for CTFE analysis Approximately 1 g of tissue was immediately removed from each organ and placed in hexane. The tissue was homogenized in hexane and the homogenate was mixed overnight. After centrifugation, the hexane layer was decanted and

analyzed Gas chromatography (GC) was used to quantify the CTFE present in blood and tissues. The chromatographic procedures used are described elsewhere in this report

RESULTS

As shown in Figure 3.7-1, GC separates CTFE into 11 major peaks occurring in two distinct groupings. For the purposes of these studies, the individual peak areas were summed to give total areas for each group, allowing quantitation of CTFE as Group 1 (lower molecular weight) and Group 2 (higher molecular weight) components. Table 3 7-1 shows the blood concentrations of Groups 1 and 2 oligomers as well as the total CTFE concentration in blood immediately after exposure and at 24 h postexposure.

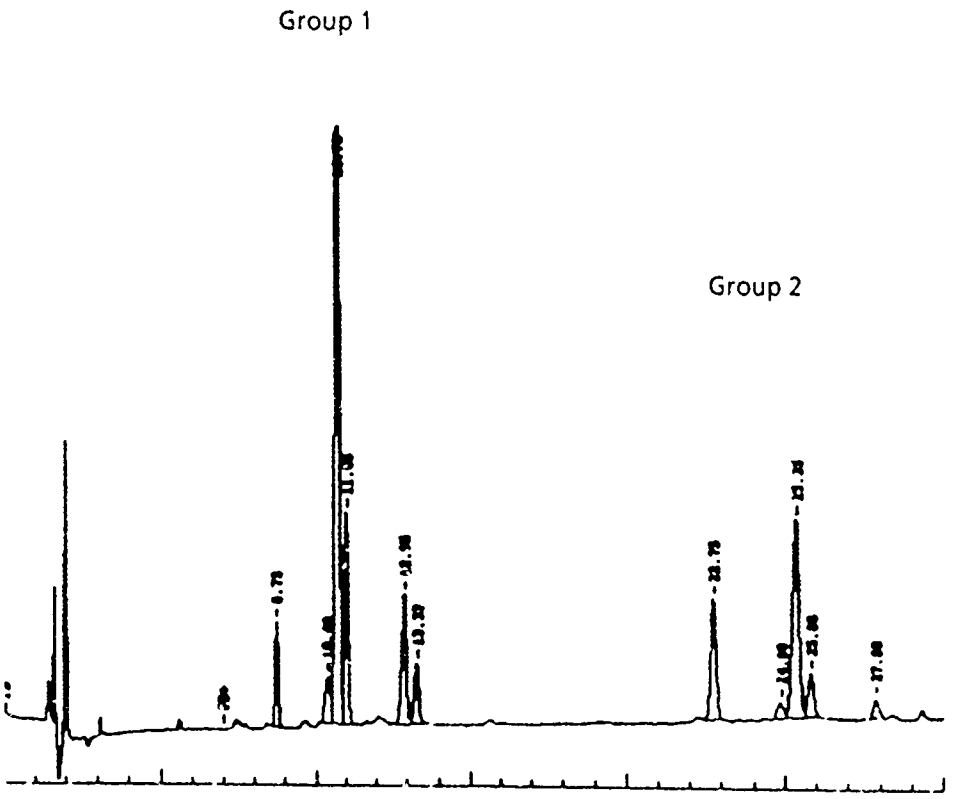

Figure 3.7-1. Gas Chromatogram of Chlorotrifluoroethylene (CTFE).

Since the animals were exposed for 6 h each day, the average blood level of CTFE was between the two values listed in Table 3 7-1. For a first approximation, the average of these two values was used as the equilibrium blood concentration in partition coefficient calculations.

TABLE 3.7-1. BLOOD CTFE CONCENTRATION IN RATS AFTER INHALATION EXPOSURE

	Group 1 ng/g	Group 2 ng/g	Total CTFE ng/g
0-h P.E.	2879	2602	5481
24-h P.E.	1291	1192	2483
Average	2100	1900	4000

Table 3.7-2 lists the calculated ratio between blood and organ CTFE concentrations for kidney, lung, liver, testes, fat, and brain for both groups; total CTFE for the four animals used in this study; and the average of these values.

DISCUSSION

For the liver, testes, and fat there appears to be little difference in the partitioning of Group 1 and Group 2 CTFE oligomers. However, this is not the case for kidney, lung, and brain. In the case of the lung, this imbalance may reflect the fact that some CTFE is deposited in liquid form as an aerosol enriched in Group 2 oligomers but leaves the lung as a vapor enriched in Group 1 oligomers These observations are reflected in the fact that Group 1 lung concentrations are lower than Group 2. In the case of the kidney, urine analysis reported elsewhere in this report indicates that Group 1 oligomers are excreted to a greater extent than Group 2. The apparent solubility of Group 1 oligomers is twice that of Group 2 oligomers in kidney tissue, suggesting that the kidney selectively absorbs Group 1 oligomers. The brain and testes data are consistent with a diffusional limitation on the rate at which Group 2 peaks move from blood into tissue.

This *in vivo* approach to estimating organ solubilities provides useful data for pharmacokinetic modeling purposes. These values are readily available from a properly planned sacrifice schedule and can be used in lieu of actual partition coefficient measurements until these later data can be obtained from the laboratory

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TABLE 3.7-2. CTFE BLOOD/TISSUE PARTITION RATIO

Animal Number	Kidney Group 1	Kidney Ratio 1	Kidney Group 2	Kidney Ratio 2	Kidney Total	Kidney Ratio T.
144	43,512[a]	20.7	22,019[a]	11.6	65,531[a]	16.4
155	35,728	17 0	20,571	10.8	56,300	14.1
162	37,258	17.7	15,797	8 3	53,055	13.3
166	46,630	22 2	20,251	10 7	66,881	16.7
Mean	40,782	19 4	19,660	10 3	60,442	15.1

Animal Number	Lung Group 1	Lung Ratio 1	Lung Group 2	Lung Ratio 2	Lung Total	Lung Ratio T.
144	13,403	6 4	33,194	17.5	46,597	11.6
155	12,690	6 0	29,802	15.7	42,492	10.6
162	23,051	11 0	50,360	26.5	73,411	18.4
166	14,757	7 0	27,451	14.4	42,208	10 6
Mean	15,975	7.6	35,202	18.5	51,177	12 8

Animal Number	Liver Group 1	Liver Ratio 1	Liver Group 2	Liver Ratio 2	Liver Total	Liver Ratio T.
144	21,658	10.3	23,237	12.2	44,895	11.2
155	25,406	12.1	28,185	14 8	53,591	13 4
162	27,003	12 9	23,267	12 2	50,270	12 6
166	27,249	13 0	18,007	9 5	45,256	11.3
Mean	25,329	12.1	23,174	12.2	48,503	12.1

Animal Number	Testes Group 1	Testes Ratio 1	Testes Group 2	Testes Ratio 2	Testes Total	Testes Ratio T.
144	9,560	4 6	7,412	3.9	16,972	4 2
155	13,977	6.7	11,874	6.2	25,851	6 5
162	10,356	4.9	7,494	3 9	17,850	4 5
166	16,120	7.7	15,115	8 0	31,235	7 8
Mean	12,503	6 0	10,474	5 5	22,977	5 7

Animal Number	Brain Group 1	Brain Ratio 1	Brain Group 2	Brain Ratio 2	Brain Total	Brain Ratio T.
144	13,167	6 3	6,265	3 3	19,431	4 9
155	16,258	7 7	7,900	4 2	24,158	6 0
162	19,145	9 1	8,305	4 4	27,450	6 9
166	21,678	10 3	9,013	4 7	30,691	7 7
Mean	17,562	8 4	7,871	4 1	25,432	6 4

Animal Number	Fat Group 1	Fat Ratio 1	Fat Group 2	Fat Ratio 2	Fat Total	Fat Ratio T.
144	756,299	360 1	897,766	472 5	1,654,065	413 5
155	712,506	339 3	837,754	440 9	1,550,260	387 6
162	712,690	339 4	831,359	437 6	1,544,049	386 0
166	757,186	360 6	952,268	501 2	1,709,454	427 4
Mean	734,670	349 8	879,787	463 0	1,614,457	403 6

[a] Values are ng/g of tissue

3.8 PHARMACOKINETICS OF CHLOROTRIFLUOROETHYLENE OLIGOMER

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INTRODUCTION

Chlorotrifluoroethylene (CTFE) oligomer, containing three and four polymer units and end capped with chlorine, is a candidate hydraulic fluid. A 90-day inhalation study of male and female Fischer 344 (F-344) rats was conducted at three exposure concentrations, 0.25, 0.5, and 1.0 mg CTFE oligomer/L air. Dose-related effects on body weight gain were noted in male rats and increased liver and kidney weights were observed in all exposure groups.

Two additional groups of male rats were exposed, one as above and one for 6 h to 0.5 mg/L. Blood and tissues were taken from these animals to obtain pharmacokinetic data that were used to support the development of a physiologically based pharmacokinetic (PB-PK) model. The PB-PK model for CTFE allows prediction of distribution of CTFE in body tissues at different exposure concentrations and, once fully validated, prediction of the pharmacokinetic behavior in man. The model then will be used for risk assessment and thereby play an important role in cost-benefit decisions on the use of CTFE.

The following report describes the exposures, tissue analyses, and development of the pharmacokinetic model.

MATERIALS AND METHODS

Exposure Regimen

Male F-344 rats, age 9 to 11 weeks, were placed in two 690-L inhalation chambers, with 12 rats/chamber, exposed 6 h a day, 5 days/week, for 13 weeks to either air alone or 0.5 mg CTFE oligomer/L. Tissue samples were obtained from the control and CTFE-exposed rats immediately and 48 h after the end of the exposure.

Two groups of age-matched rats (four each) were exposed for a single 6-h exposure to 0.5 mg CTFE oligomer/L. These were sacrificed for tissue samples at 48 h and 14 days following exposure.

Blood samples were taken via the lateral tail vein from all rats at intervals following exposure and analyzed for CTFE. Urine and feces were collected daily from one exposed and one control group for a period of two weeks following conclusion of the 30-day study for CTFE analysis. The urine samples were also analyzed for fluoride concentration. Kidney, lung, liver, testes, brain, and fat were collected from all rats at sacrifice for CTFE analysis.

Analysis of Biologic Samples

Blood was collected for CTFE analysis in 79 8 µL capillary tubes via the lateral tail vein. Blood samples were transferred into 20 mL scintillation vials containing 5.0 mL hexane To reduce CTFE vaporization, the transfer occurred below the surface Extraction of CTFE was accomplished using an Evapotec® mixer for 2 h.

To avoid analysis interference, no refrigerant or preservative was used during the collection of 24-h urine samples for CTFE analysis The samples were transferred into hexane following the same procedure as described for blood The CTFE was extracted using an Evapotec® mixer for 1 h.

Fluoride excretion via the urine was determined by ion-specific electrodes using the method of Neefus et al., (1970). To avoid analysis interference, preservatives were not used in the 24-h urine collection. The weight of the urine was converted to volume using a value of 1.06 g/mL for urine density. The fluoride concentration of the urine was determined by diluting the urine by 50% with a total ionic strength urinary buffer (Neefus et al , 1970), and measuring the resulting cell potential with an ion selective electrode The instrument was calibrated by measuring the cell potential of standard solutions using the same buffer and dilution conditions

A weighed sample of feces was collected daily over a two-week postexposure period. The CTFE was extracted in hexane while shaking overnight After centrifugation the hexane layer was removed and stored at -70°C until analyzed

Bone and tooth fluoride concentrations were determined using the method of Singer and Armstrong (1968) The sample (bone or tooth) was cleaned by immersion in a 10% solution of papain until void of extraneous tissue, then rinsed in deionized water and dried at 100°C in a vacuum oven overnight A mortar and pestle was used to break the bones/teeth into small pieces, which were then ground to a fine powder in a ball mill. The sample powder was then weighed in a nickel crucible and ashed at 550°C The ash was dissolved in dilute hydrochloric acid, adjusted to pH 4.7 by titrating with 0.05 M sodium acetate, brought up to volume, and then measured by fluoride electrode. The instrument was calibrated using standard fluoride solutions and an acetate buffer

Tissues collected for CTFE analysis were weighed and then maintained in hexane on ice until homogenization The tissues were homogenized then mixed overnight Following centrifugation, the hexane layer was stored at -70°C, but allowed to return to room temperature before analysis Tissue extracts were diluted with hexane to reduce the CTFE in the samples to less than 500 ng/mL for analytical purposes Lung, liver testes, and brain extracts were diluted to 5%, kidney to 2%, and fat to 0.1%.

The assumption of a theoretical 100% extraction efficiency for CTFE in blood, urine, and tissue was based on studies conducted using a hexane solution of CTFE and allowing that solution to be intimately mixed with biological samples for sufficient time to permit uptake of the CTFE by tissues

The hexane solutions were analyzed to determine losses. There were no measurable losses of CTFE to the tissues.

Gas Chromatographic Analysis

A gas chromatograph (GC) equipped with an electron capture detector (ECD) was used to analyze the biologic samples. A Nelson® integration system was programmed to handle the GC output. CTFE-hexane solution standards were used to quantify the ECD signal. The usable standard range was between 1 and 500 ng/mL.

Model Development

A PB-PK model was written in Simusolv, (Mitchell and Gauthier Associates, Concord, MA), a FORTRAN-based continuous simulation language with optimization capabilities and run on a VAX 8530 (Digital Equipment Corp., Maynard, MA). The general form of the model follows that of Ramsey and Andersen (1984) Fat, lung, liver, kidney, brain, testes, and rapidly perfused and slowly perfused organ groups were described The CTFE oligomer was separated into two distinctive groups of chromatographic peaks, referred to as Group I and Group II, respectively. This modeling effort has focused on Group I oligomers, which existed in the chambers only as a vapor. Group II oligomers, which existed as vapor and aerosol, have not been modeled as yet. Partition coefficients were estimated from tissue:blood concentration ratios immediately after the 90-day exposure.

RESULTS

GCs of CTFE showed two distinct groups of peaks Group I had five peaks with retention times between 10 and 14 min. Group II also had five peaks with retention times between 22 and 28 min. The average exposure chamber concentration for the animal group from which pharmacokinetic data were collected was 0.48 mg/L. Apportioning the areas for the two groups of peaks resulted in Group I and Group II concentrations of 0.35 and 0.13 mg/L, respectively. CTFE was also chromatographed using a thermal conductivity detector (TCD) that normally responds to compounds in proportion to their concentration The TCD analysis was similar to that obtained from ECD.

The results of the analyses of CTFE in blood samples taken following a single, 6-h exposure to 0.5 mg CTFE/L are provided in Table 3.8-1. The results following repeated exposure are provided in Table 3.8-2. CTFE concentration in blood samples from the singly exposed rats gradually decreased through seven days, after which the concentration was below the detection limits. The CTFE concentrations found in the repeated-exposed rat blood samples taken immediately following exposure were twice that of the singly exposed rats This difference in CTFE concentration increased to 13 times by 48 h. By 21 days postexposure, the CTFE concentration in the repeated-exposed rat blood samples was minimal. No CTFE was found in blood samples taken from control rats

TABLE 3.8-1. BLOOD CONCENTRATIONS (ng CTFE/mL) IN GROUP I, GROUP II OLIGOMERS, AND TOTAL CTFE FROM RATS FOLLOWING A SINGLE 6-h EXPOSURE TO CTFE

Target Sample Time	Actual Time (h)	GC Group I Oligomers	GC Group II Oligomers	Total
Immediately	0.05	1,563	1,135	2,698
Following	0.05	1,843	899	2,742
Exposure	0.07	2,672	2,159	4,830
	0 08	1,753	1,114	2,868
	0.10	1,768	1,007	2,776
	0.12	2,033	1,203	3,236
	0.15	1,651	1,055	2,706
	0.18	1,964	1,390	3,354
1 h	0.47	1,503	925	2,428
Following	0.55	1,171	876	2,048
Exposure	0 55	1,286	801	2,087
	0 58	1,484	1,009	2,493
	0.97	418	681	1,099
	1 00	488	714	1,203
	1.02	540	792	1,332
	1 08	457	697	1,154
1 Day	23.98	325	171	496
Following	24.00	304	161	465
Exposure	24.00	270	132	402
	24 03	317	304	621
2 Days	47.50	149	0[a]	149
Following	47 52	152	0	152
Exposure	47.53	153	0	153
	47.57	153	0	153
7 Days	167 52	171	0	171
Following	167.58	87	0	87
Exposure	167 62	67	0	67
	167 77	76	0	76
14 Days	335 20	0	0	0
Following	335.22	0	0	0
Exposure	335.25	0	0	0
	335 33	0	0	0

[a] 0 = Concentrations below detection limits

TABLE 3.8-2. BLOOD CONCENTRATIONS (ng CTFE/mL) OF GROUP I, GROUP II OLIGOMERS, AND TOTAL CTFE FOLLOWING REPEATED INHALATION EXPOSURE TO CTFE

Target Sample Time	Actual Time (h)	GC Group I Oligomers	GC Group II Oligomers	Total
Immediately	0.05	3,313	2,949	6,262
Following	0.08	3,103	2,723	5,826
Exposure	0.08	2,829	2,317	5,145
	0.08	3,460	2,867	6,327
	0.12	3,259	2,774	6,033
	0.12	3,419	3,439	6,858
	0.13	2,912	2,448	5,359
	0.13	3,055	2,460	5,515
	0.15	3,052	2,592	5,644
	0.17	2,939	2,474	5,413
	0.18	2,830	2,543	5,373
	0.18	2,878	2,619	5,497
1 h	0.53	2,533	2,148	4,681
Following	0.63	2,742	2,393	5,135
Exposure	0.65	2,148	1,718	3,866
	0.67	2,983	2,698	5,680
3 h	2.88	1,749	1,906	3,656
Following	2 90	1,738	1,632	3,370
Exposure	2.93	2,208	2,446	4,653
	2.95	1,819	1,746	3,566
1 Day	23 57	1,275	1,126	2,401
Following	23.58	1,614	1,692	3,306
Exposure	23.63	1,373	996	2,369
	23.65	1,186	1,216	2,402
2 Days	47.53	1,099	962	2,061
Following	47.53	1,029	1,054	2,083
Exposure	47.58	1,209	1,197	2,407
	47.60	1,019	904	1,923
7 Days	167 60	451	408	859
Following	167 63	430	590	1,020
Exposure	167 68	437	522	958
	167.72	323	378	701
21 Days	503 10	0[a]	0	0
Following	503.12	96	0	96
Exposure	503.13	89	0	89
	503.20	0	243	243

(Continued)

TABLE 3.8-2. (Continued)

Target Sample Time	Actual Time (h)	GC Group I Oligomers	GC Group II Oligomers	Total
35 Days	839.20	0	0	0
Following	839 22	0	0	0
Exposure	839.23	0	0	0
	839.25	0	0	0
49 Days	1,176.20	0	0	0
Following	1,176 23	0	138	138
Exposure	1,176 23	0	0	0
	1,176 27	0	0	0

[a] 0 = Concentrations below detection limits

Only Group I oligomers were found in the urine samples Urine from the repeated-exposure rats initially contained approximately nine times the CTFE concentration found in that of the singly exposed rats (Table 3.8-3). CTFE was found in urine samples for three days following a single exposure and for 13 days following repeated exposures CTFE was not detected in urine collected from control rats.

TABLE 3.8-3. CTFE CONTENT[a] OF URINE FROM RATS EXPOSED TO 0.5 mg CTFE/L

The data provided indicate the mean amount of CTFE excreted during a 24-h period.

Following Single Inhalation		Following Repeated Inhalation	
Time (Days)	Total CTFE (ng)	Time (Days)	Total CTFE (ng)
1	204 ± 30	1	1,780 ± 194
2	107 ± 36	2	1,966 ± 287
3	301 ± 21	3	1,103 ± 176
4	88[b]	4	840 ± 58
5	0[c]	5	545 ± 62
6	0	6	602 ± 99
7	0	7	543 ± 64
8	0	8	877 ± 120
9	0	9	516 ± 69
		10	392 ± 34
		11	326 ± 29
		12	222 ± 74
		13	183 ± 119
		14	57[b]
		21	77[b]
		28	0
		35	0
		42	0
		49	0

[a] Mean ± S E M , N = 4
[b] Value for one animal only, the other three had no detectable CTFE
[c] 0 = Concentration below detection limits

An increase ($p<0.01$) in total inorganic fluoride in the urine of the repeated exposure rats was noted for eight weeks postexposure (Table 3 8-4). The inorganic fluoride concentration of the control rat urine remained relatively stable throughout the testing period. The results of inorganic fluoride analysis from urine samples taken from rats following a single exposure were not significantly different from the control data

TABLE 3.8-4. RAT URINE TOTAL INORGANIC FLUORIDE[a] (µg FLUORIDE/24 h)

Days Postexposure	Control	Test
1	52	131[b]
2	43	161[b]
3	48	164[b]
4	50	134[b]
5	38	104[b]
6	46	128[b]
7	44	124[b]
8	44	126[b]
9	40	129[b]
10	43	130[b]
11	52	136[b]
12	46	133[b]
13	44	124[b]
14	45	116[b]
21	47	122[b]
28	49	96[b]
35	51	86[b]
42	51	91[b]
49	62	82
56	52	74[b]
63	48	64
70	53	66[c]
77	59	68
84	54	69[c]
91	64	71

[a] Mean, N = 4
[b] Compared with control, $p < 0.01$, as determined by the Multivariate Analysis of Covariance for Repeated Measures Test
[c] Compared with control, $p < 0.05$, as determined by the Multivariate Analysis of Covariance for Repeated Measures Test

Feces – CTFE Analysis

GCs of CTFE extracted from feces showed poor resolution and low amplitude of peaks No useful information was obtained

Bone and Tooth – Fluoride Analysis

The results of the analyses of inorganic fluoride revealed that concentrations in bones of rats removed after a single 6-h exposure were not different from those of the control rats' bones (Table 3 8-5). However, a slight but statistically significant increase was noted in the inorganic fluoride concentrations of bones taken from rats following repeated exposures to 0 5 mg CTFE/L The results of incisor fluoride analysis were so variable that meaningful statistical analysis could not be conducted

TABLE 3.8-5. MEAN[a] BONE INORGANIC FLUORIDE CONCENTRATIONS (µg FLUORIDE g BONE) OF MALE RATS EXPOSED TO 0.5 MG CTFE/L

Test Regimen	Inorganic Fluoride Concentration
Control	255 ± 43
Repeat Exposure[b] Sacrificed Immediately	593 ± 74[c]
Repeat Exposure[d] Sacrificed at 48 h	582 ± 73[c]
Single Exposure Sacrificed at 48 h	326 ± 55
Single Exposure Sacrificed at 14 Days	279 ± 61

[a] Mean ± S E M , measurements were made using a single femur from each rat A minimum of five measurements per rat were made
[b] 90 days on study
[c] Compared with control, $p < 0.05$, as determined by the Multivariate Analysis of Covariance for Repeated Measures Test
[d] 84 days on study

Tissues – CTFE Analysis

The results of the analysis of CTFE in rat tissues taken following a single, 6-h exposure and following repeated exposures are provided in Tables 3 8-6 and 3 8-7, respectively There was wide variation in tissue CTFE concentrations from the single exposure rats sacrificed at 48 h postexposure Liver, testes, and brain tissues were lowest in CTFE concentration Kidney, lung, and fat were, respectively, 3, 6, and 40 times greater in CTFE concentration than the other analyzed tissues A substantial decrease in tissue CTFE concentration occurred in the single-exposure rats held for 14 days Although Group I and Group II oligomers decreased, Group I compounds decreased more rapidly The concentration of Group I oligomers in the fat also decreased with time The data on Group II oligomer concentration in fat suggest an increase from 2 to 14 days postexposure This result seems unlikely, and the data on Group II oligomers in fat must, therefore, be viewed with suspicion

The CTFE tissue concentration of the repeated-exposure rats decreased 50% in most tissues (except for fat) when the rats were held for 48 h postexposure Group I oligomers decreased more rapidly than the Group II oligomers The more rapid Group I loss continued as rat tissue examined 105 days postexposure showed the loss of all Group I oligomers and the remaining CTFE was detected as Group II peaks By 105 days the total CTFE concentration in fat had decreased 94% from its highest concentration immediately following exposure Control rat tissues did not contain CTFE. An interfering peak found in lung tissue Group II oligomer peaks was also found in control rats and was subtracted from the reported Group II oligomer and total CTFE concentrations.

TABLE 3.8-6. TISSUE CONCENTRATIONS[a] (ng CTFE/g), GROUP I, GROUP II OLIGOMERS, AND TOTAL[b] CTFE FROM RATS FOLLOWING A SINGLE 6-h EXPOSURE TO CTFE

		Tissue Removed Postexposure	
		48 h	14 Days
Kidney:	Group I	2,585 ± 362	618 ± 106
	Group II	661 ± 326	359 ± 122
	Total	3,246 ± 663	977 ± 196
Lung:	Group I	4,247 ± 754	1,109 ± 53
	Group II	3,700 ± 629	1,011 ± 84
	Total	7,947 ± 1361	2,120 ± 113
Liver:	Group I	604 ± 108	0[d]
	Group II	572 ± 64	0
	Total	1,176 ± 170	0
Testes:	Group I	523 ± 52	181 ± 15
	Group II	571 ± 26	0
	Total	1,093 ± 70	181 ± 15
Brain:	Group I	738 ± 57	0
	Group II	567 ± 96	0
	Total	1,305 ± 105	0
Fat:	Group I	44,718 ± 1286	25,870 ± 2,943
	Group II	2399[c]	18,023 ± 4,410
	Total	47,117 ± 2573	43,893 ± 7,019

[a] Mean ± S E M , N = 4
[b] Values assuming 100% CTFE extraction from tissue
[c] Value for one animal only, the other three had no detectable CTFE
[d] 0 = Concentration below detection limits

TABLE 3.8-7. TISSUE CONCENTRATIONS[a] (ng CTFE/g) OF GROUP I, GROUP II OLIGOMERS, AND TOTAL[b] CTFE FROM RATS FOLLOWING REPEATED INHALATION EXPOSURE TO CTFE

		Tissue Removed Postexposure		
		0 Days[c]	2 Days[d]	105 Days[c]
Kidney:	Group I	40,782 ± 2,576	16,227 ± 365	0[e]
	Group II	19,660 ± 1,344	16,689 ± 1,371	1,970 ± 46
	Total	60,442 ± 3,404	32,916 ± 1,384	1,970 ± 46[f]
Lung:	Group I	15,975 ± 2,397	8,834 ± 2,223	0
	Group II	35,202 ± 5,188	20,989 ± 4,981	541 ± 45
	Total	51,117 ± 7,479	29,823 ± 7,204	541 ± 45[f]
Liver:	Group I	25,329 ± 1,290	9,356 ± 778	0
	Group II	23,174 ± 2,078	14,784 ± 1,560	495
	Total	48,503 ± 2,093	24,139 ± 2,327	495[g]
Testes:	Group I	12,503 ± 1,542	4,740 ± 492	0
	Group II	10,474 ± 1,865	7,124 ± 628	270
	Total	22,977 ± 3,401	11,864 ± 1,105	270[f,g]
Brain:	Group I	17,562 ± 1,836	6,033 ± 161	0
	Group II	7871 ± 583	6,453 ± 265	0
	Total	25,433 ± 2,404	12,486 ± 250	0
Fat:	Group I	734,670 ± 12,745	738,268 ± 33,689	0
	Group II	879,787 ± 28,415	990,291 ± 43,707	100,475 ± 15,536
	Total	1,614,457 ± 40,448	1,728,559 ± 75,308	100,475 ± 15,536[f]

[a] Mean ± S E M , N = 4
[b] Values assuming 100% CTFE extraction from tissue
[c] 90 Exposures
[d] 84 Exposures
[e] 0 = Concentration below detection limits
[f] N = 2
[g] Value represents only one animal, tissue concentrations of remaining animals below detection limits

Partition Ratio

The blood and tissue CTFE concentrations in 90 day exposed rats were used to calculate a partition ratio It was assumed that the test rats were exposed to CTFE for a long enough time and that the CTFE in tissues had approached an equilibrium with the blood The partition ratios were calculated from CTFE concentrations of test rat tissue taken immediately after the exposure end divided by the blood concentrations (Table 3 8-8)

TABLE 3.8-8. TISSUE CONCENTRATION[a] (ng CTFE/g) AND PARTITION RATIO (TISSUE/BLOOD) ASSUMING 100% CTFE EXTRACTION FROM THE TISSUE

	Mean Tissue Concentration		Ratio	
Tissue	Group I	Group II	Group I	Group II
Kidney	40,782	19,660	12 8	7.2
Lung	15,975[b]	35,202[b]	5 0	13 0
Liver	25,3[illegible]9	23,174	8 0	8.5
Testes	12,503	10,474	3 9	3 9
Brain	17,562	7,871	5.5	2.9
Fat	734,670	879,787	231 3	324 2

[a] Tissue data from animals exposed 90 days and sacrificed immediately
[b] Lung concentrations less the control average

Possible Metabolites

Analysis of urine and kidney extracts showed a significant increase in the relative percent area of the first peak identified as a CTFE oligomer in standards This increase was not observed in other tissues to the same degree, nor was this peak present in control samples of tissue. For these reasons, the peak was identified as a possible metabolite Since there were 11 discrete peaks identified in the standards and this peak was obviously not totally composed of starting material, it was excluded from the total area for quantitation Although the disproportionation occurred only in selected extracts, the peak was eliminated from interpretation of all chromatograms in the interest of consistency

Mass spectral data from electron impact and chemical ionization mass spectrometry tentatively identified the possible metabolite as $C_5F_7Cl_5$ In the absence of a molecular ion by either technique, a definitive identification is not possible The retention time of this material and the available mass spectral data are consistent with the identification

Model Development

The fat compartment was modeled with a diffusion limitation that was proportional to a weight-scaled diffusion constant and to blood flow to the compartment Metabolism was assumed to be first order An estimate of the rate of metabolism was made from the amount of fluoride in the urine collected 24 h after the end of the 90-day exposure There was an average of 99 µg fluoride in the urine collected from male rats weighing about 300 g This is equivalent to 4 125 µg fluoride excreted per h, or $2\ 2 \times 10^{-7}$ mol fluoride per h ($1\ 9 \times 107$ µg fluoride per mol) Assuming 1 5 mol fluoride produced per mol of CTFE oligomer, $1\ 47 \times 10^{-7}$ mol CTFE were metabolized per h On a mola. basis, 80% of the CTFE oligomer was contained in Group I peaks, thus $1\ 18 \times 10^{-7}$ mol or 05 mg of Group I oligomers were metabolized per h The first order rate constant was determined to be

0.131 h^{-1} by iteratively running the simulation under equilibrium conditions until a metabolic rate of .05 mg h^{-1} was achieved.

Simulations (solid line) of the acute exposure (Figures 3 8-1 through 3 8-8) and of the subchronic exposure (Figures 3 8-9 through 3 8-17) are shown co-plotted with actual data (squares) collected during the postexposure period of each study Tissue data were collected two days and two weeks postexposure for the acute study and immediately, two days, and 105 days postexposure for the subchronic study Blood and urine samples were collected more frequently

DISCUSSION

The model does a reasonable job of fitting both the single and 90-day exposures However, the goodness of fit could be improved if certain additional data were available Because rapid decreases in concentrations of CTFE occurred in the first two days (see blood concentrations, Figures 3 8-2 and 3.8-10), tissue concentration data obtained immediately after the acute exposure would allow a better fit of the model. Fitting the subchronic data would be aided with additional data points taken at two weeks postexposure A second source of data uncertainty relates to the partition coefficients. These were obtained from the ratios of measured tissue and blood concentrations immediately after the subchronic exposure Since rapid declines occurred in concentration immediately postexposure, any lags in sampling between tissues and blood would produce an error in the partition coefficient calculations Furthermore, the significance of the lag would be exaggerated in any tissues with a diffusional limitation for the passage of CTFE The simulation indicated that such a limitation occurred in fat The lung.air partition coefficient was determined empirically by iteration to find a best fit of the simulation to the data Direct determinations of partition coefficients should be made for all tissues described in the model

Results were not presented for the second group of higher molecular weight oligomers because part of the exposure to Group II oligomers was in aerosol form It was uncertain how much aerosol actually entered and was absorbed by the rats, because exposure occurred both by inhalation and by oral ingestion from grooming of droplets deposited on the animals' pelage The model has not yet been modified to account for the aerosol exposure Subsequent experiments will be conducted at lower concentrations where aerosol generation will be avoided entirely

Postexposure inorganic fluoride content of urine from animals that were subchronically exposed to CTFE were elevated compared to urine of control animals (Table 3 8-4) The concentrations diminished to those of controls over a 91 day period The excess fluoride is presumed to be the result of CTFE biotransformation Since the ends of the oligomers were capped with chlorine, there were either one or two fluorides available for release after the initial oxidation of the oligomer There would thus be an average of 1 5 mol of fluoride released for every mol of CTFE

metabolized Approximately half of the fluoride released into the circulation would have been excreted in the urine, with the remainder being stored in calcified tissue (Van Gelder, 1976). Figure 3.8-17 shows the fluoride (corrected for control values) in the urine up to 91 days postexposure. These are co-plotted with the simulation.

The model indicated that CTFE pharmacokinetics are sensitive to the fat:blood and blood:air partition coefficients and to diffusional restriction on CTFE movement in fat. Moreover, the model showed that although the 90-day exposure was intermittent, fat concentrations of CTFE increased over time to the point where fat storage of CTFE drove continuous, 24 h/day, blood and tissue exposure that was modulated upward during the daily inhalation exposure. This latter observation is probably relevant to hepatic lesions that developed during the 90-day study and that are described elsewhere. In addition to providing insights to the pharmacokinetic behavior of CTFE, this study also illustrates PB-PK modeling of mixtures of structurally similar materials.

Results from this experiment are being used to design the sampling strategy for the next 90-day CTFE inhalation study Additional sampling points will be selected to give a better experimental description of the change in concentration of CTFE in tissues postexposure. Partition coefficients will be determined using an *in vitro* method

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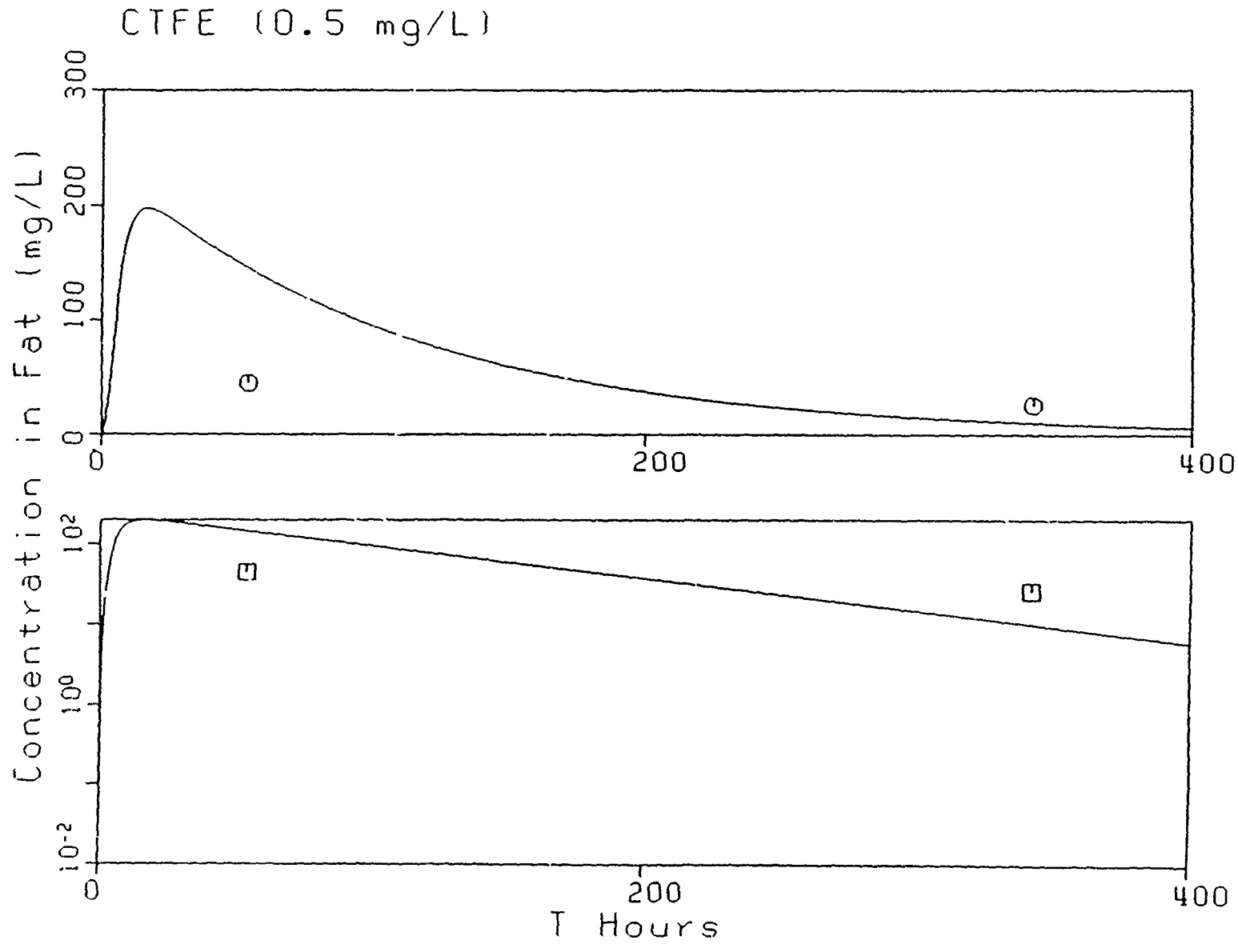

Figure 3.8-1. Concentration of CTFE (Group I Oligomers) in Fat during and after Single 0.5 mg/L, 6-h Exposure. *Solid lines represent simulations, points represent data* Linear plot, upper curve and hexagonal points Log plot, lower curve and square points

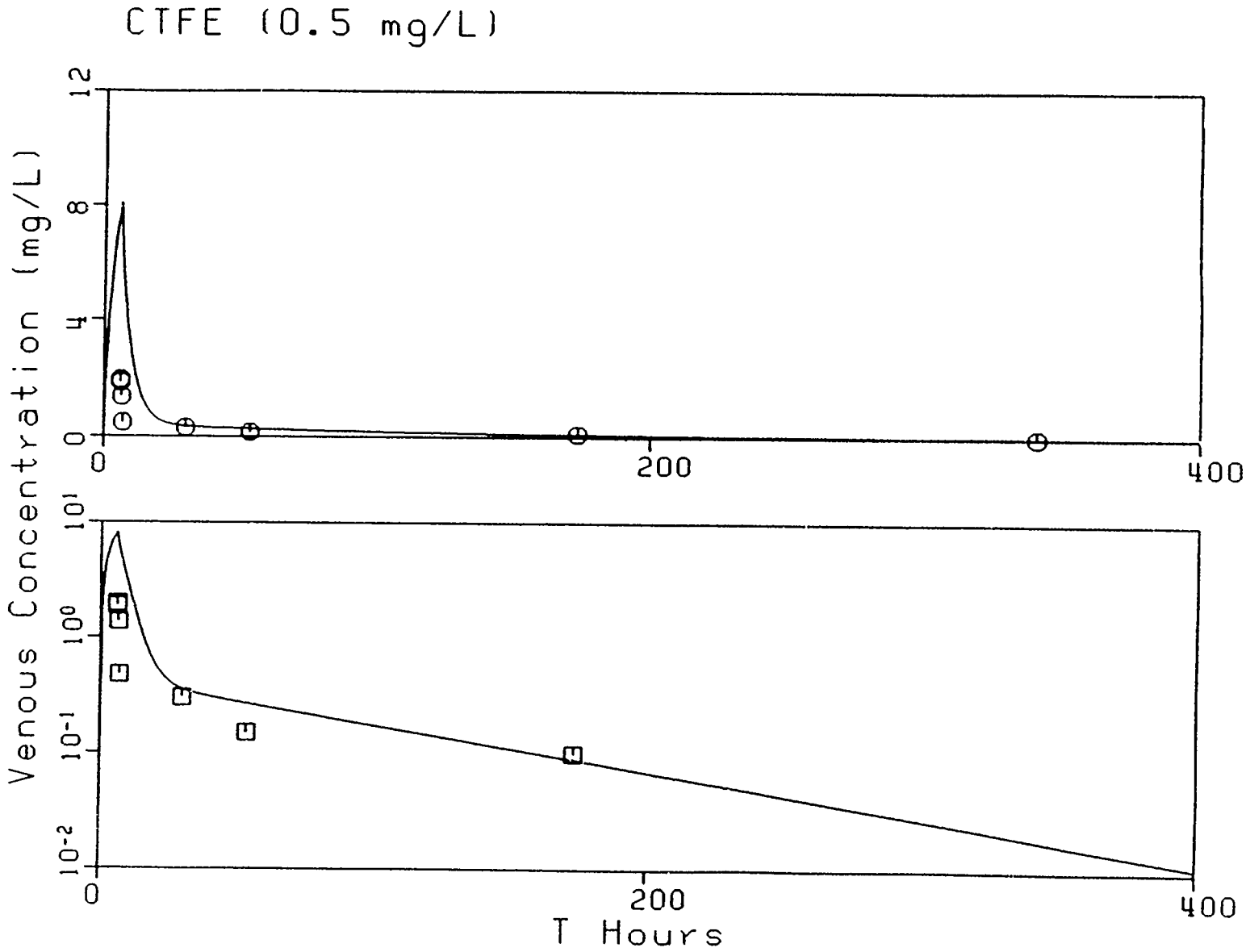

Figure 3.8-2. Concentration of CTFE (Group *i* Oligomers) in Venous Blood during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square

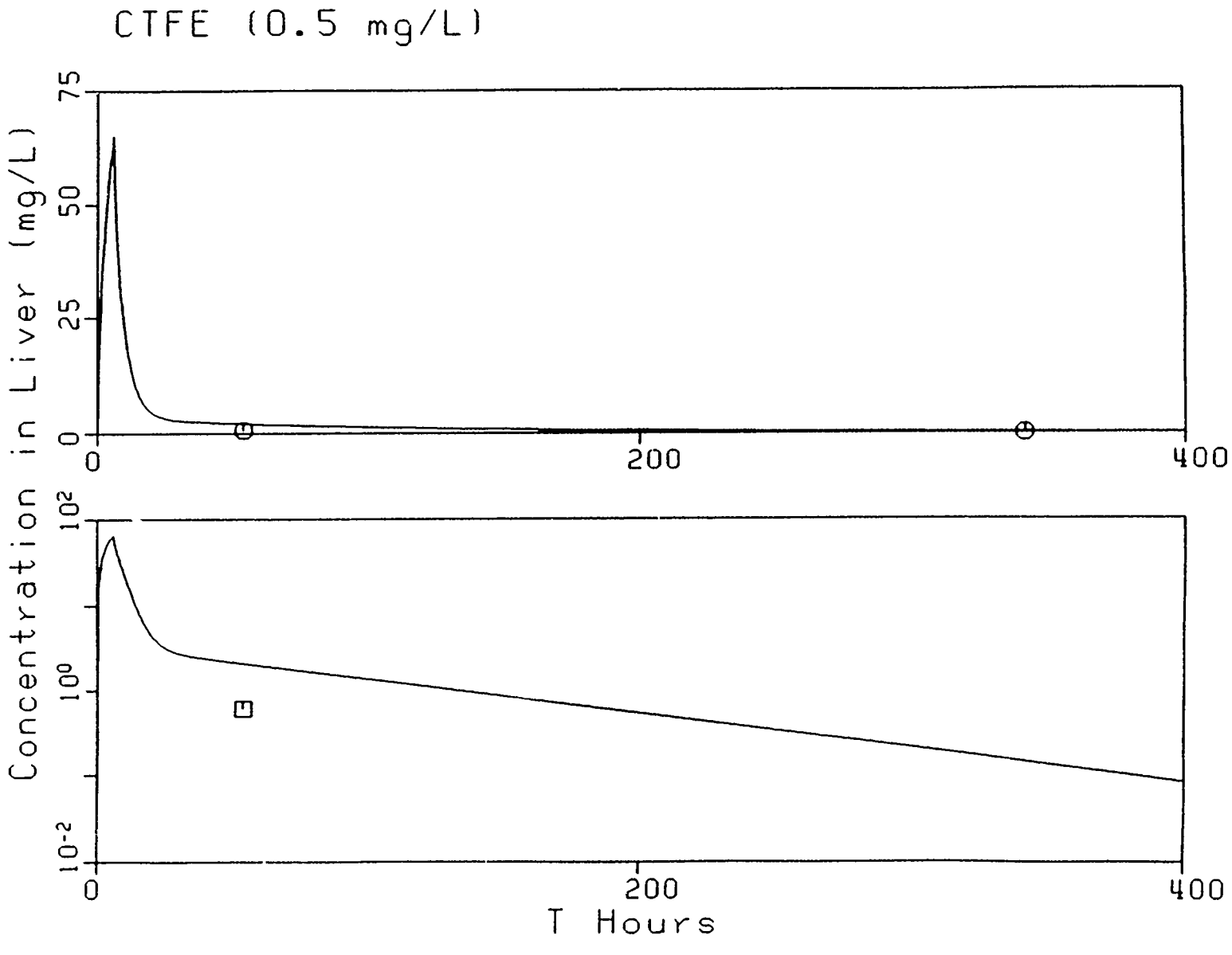

Figure 3.8-3. Concentration of CTFE (Group I Oligomers) in Liver during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

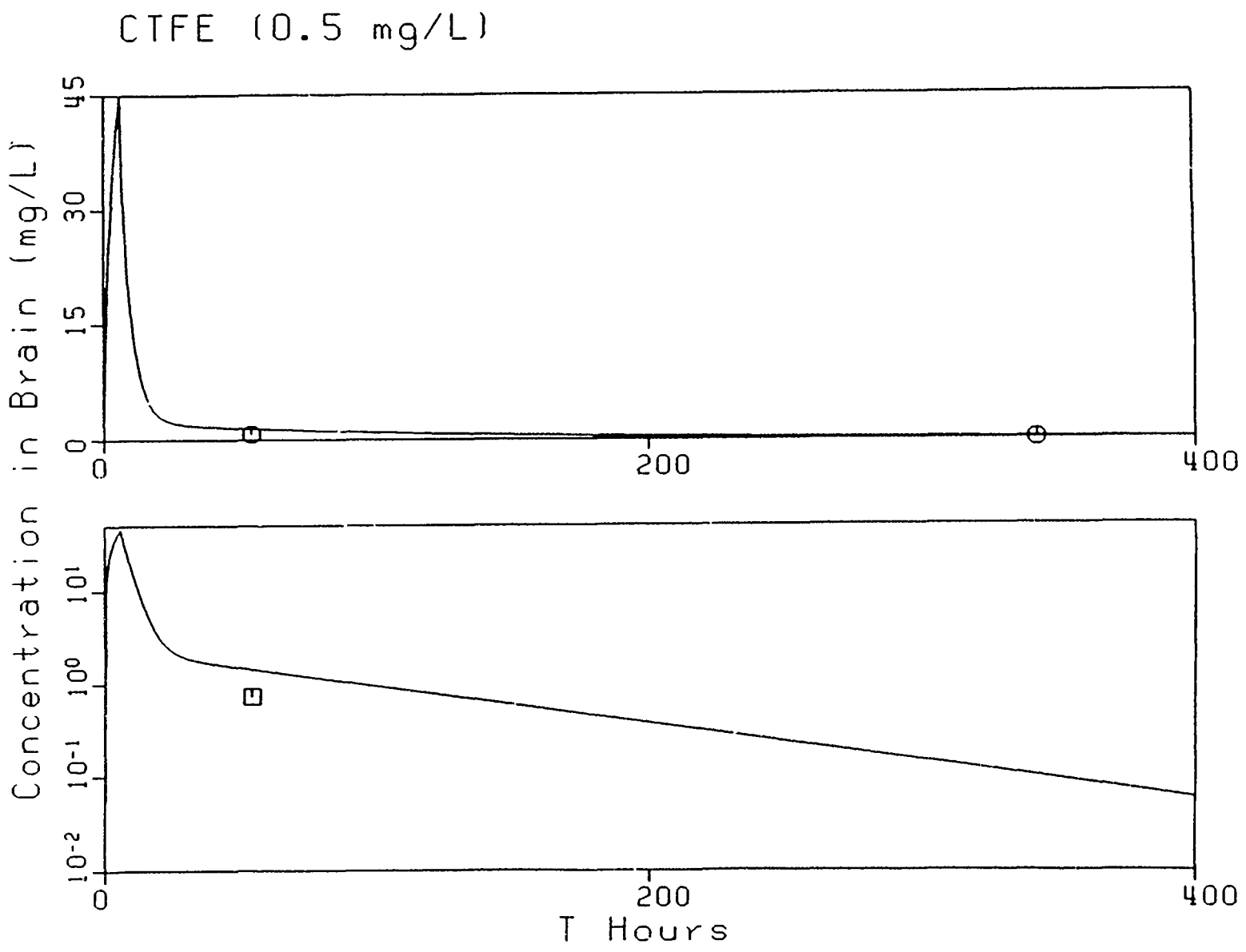

Figure 3.8-5. Concentration of CTFE (Group I Oligomers) in Brain during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points

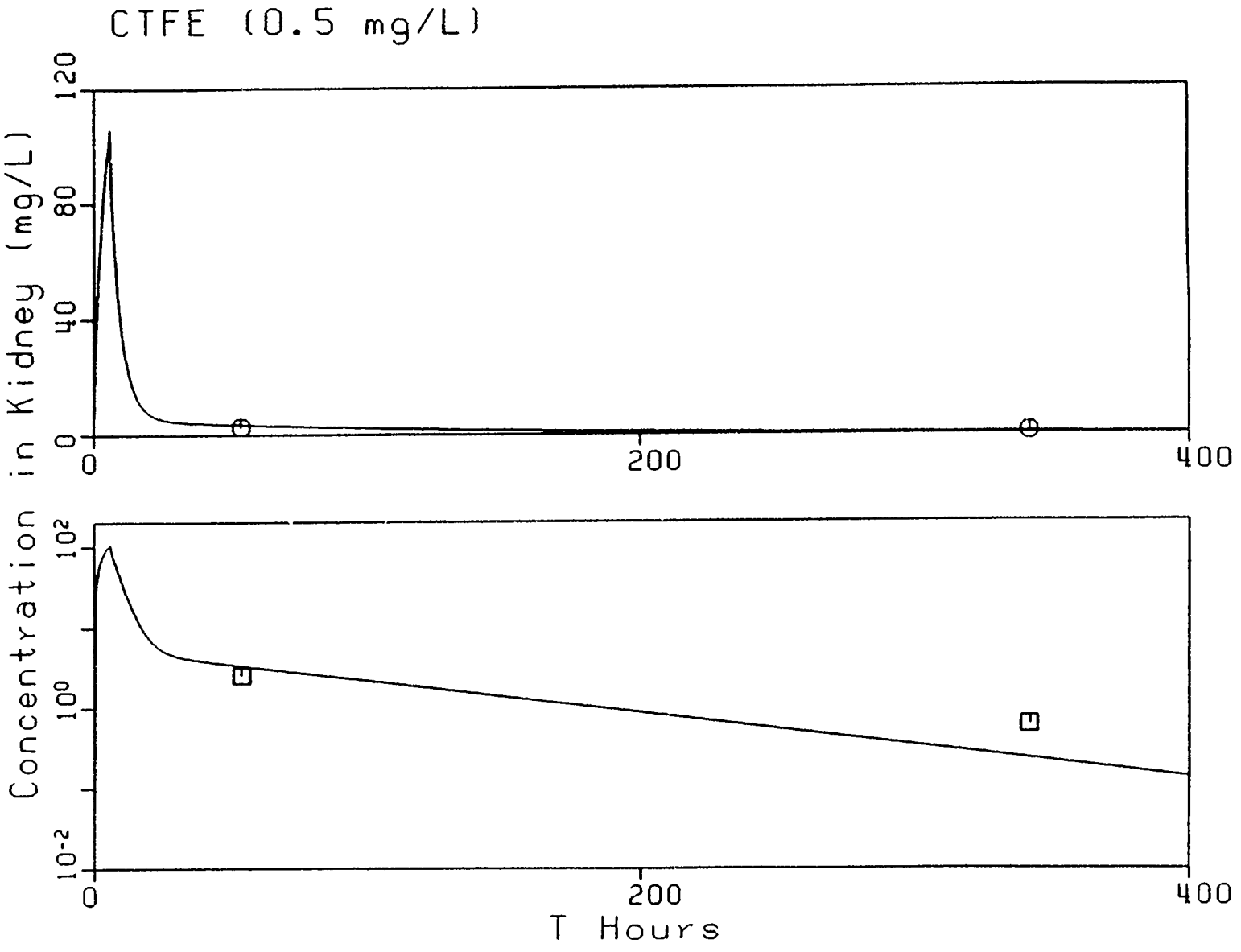

Figure 3.8-4. **Concentration of CTFE (Group I Oligomers) in Kidney during and after Single 0.5 mg/L, 6-h Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points Log plot, lower curve and square points

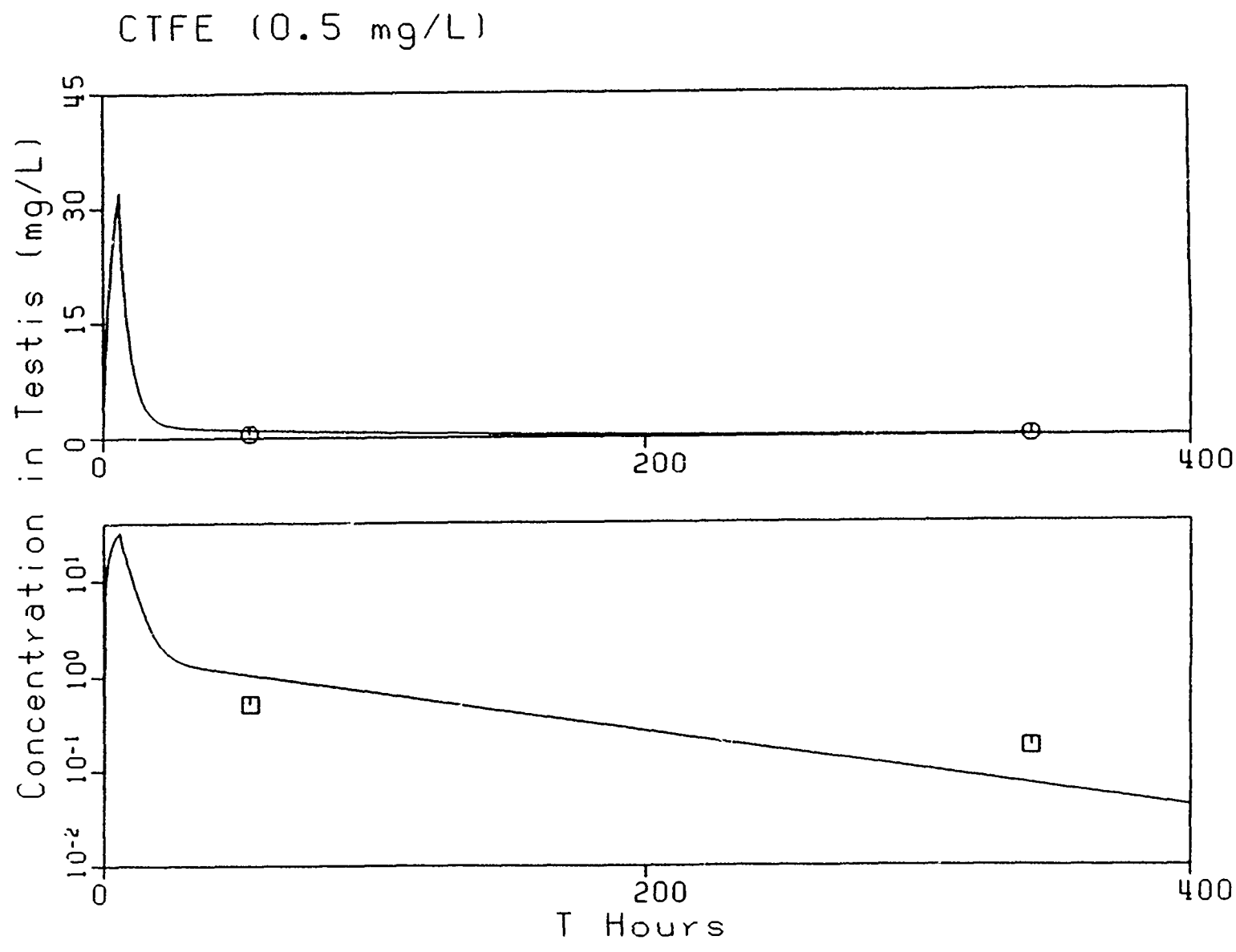

Figure 3.8-6. Concentration of CTFE (Group I Oligomers) in Testis during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points

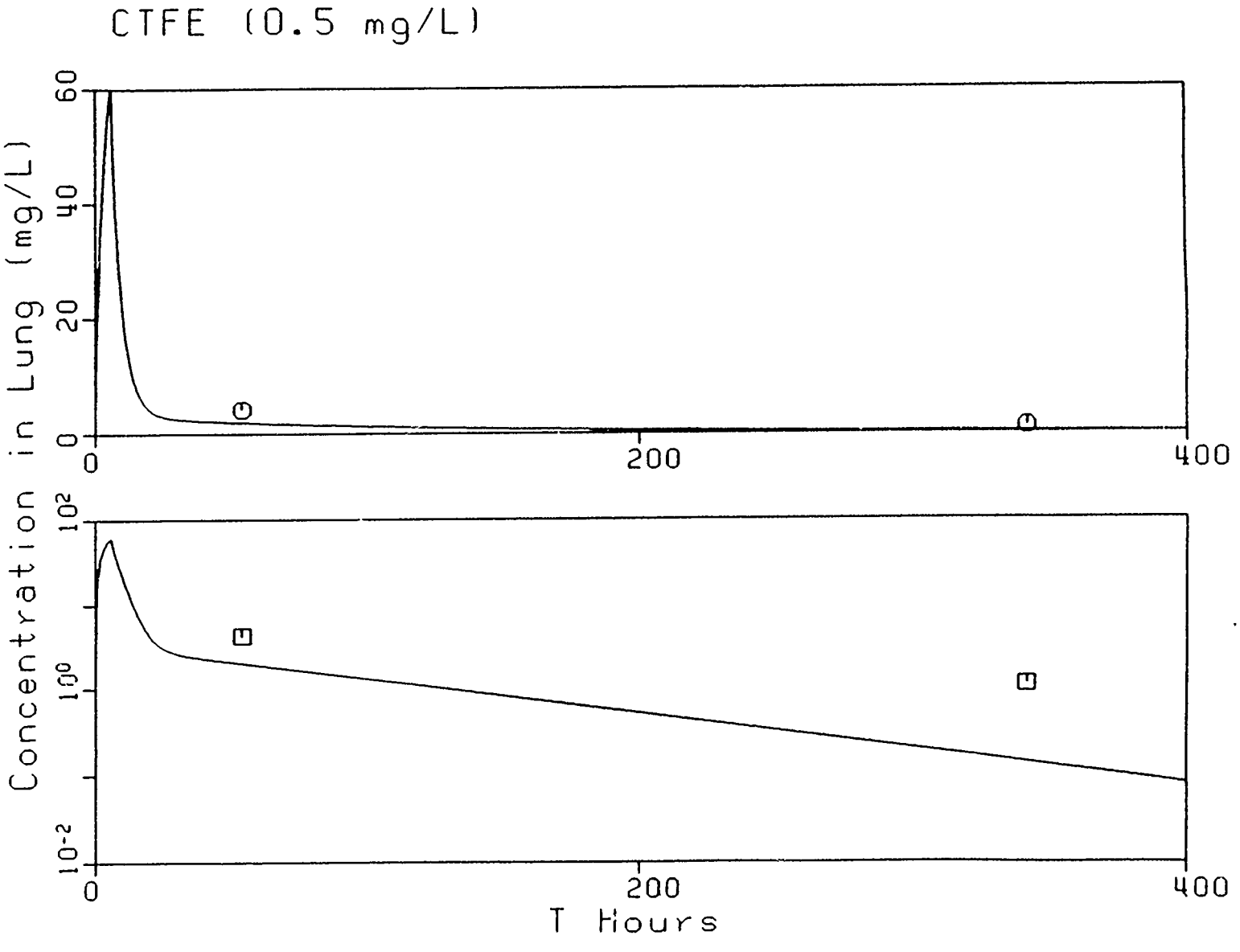

Figure 3.8-7. Concentration of CTFE (Group I Oligomers) in Lung during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

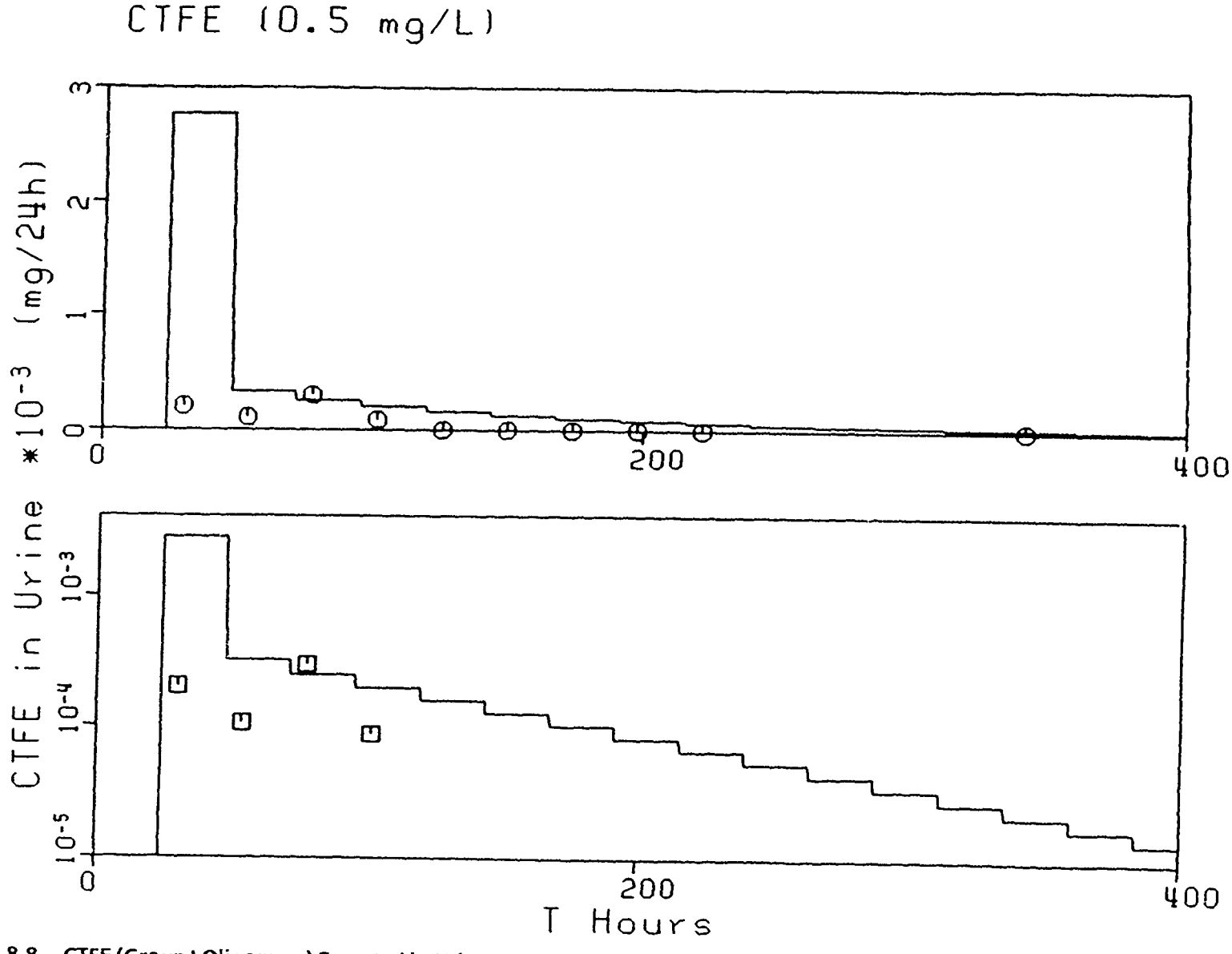

Figure 3.8-8. CTFE (Group I Oligomers) Excreted in Urine Every 24 h during and after Single 0.5 mg/L, 6-h Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

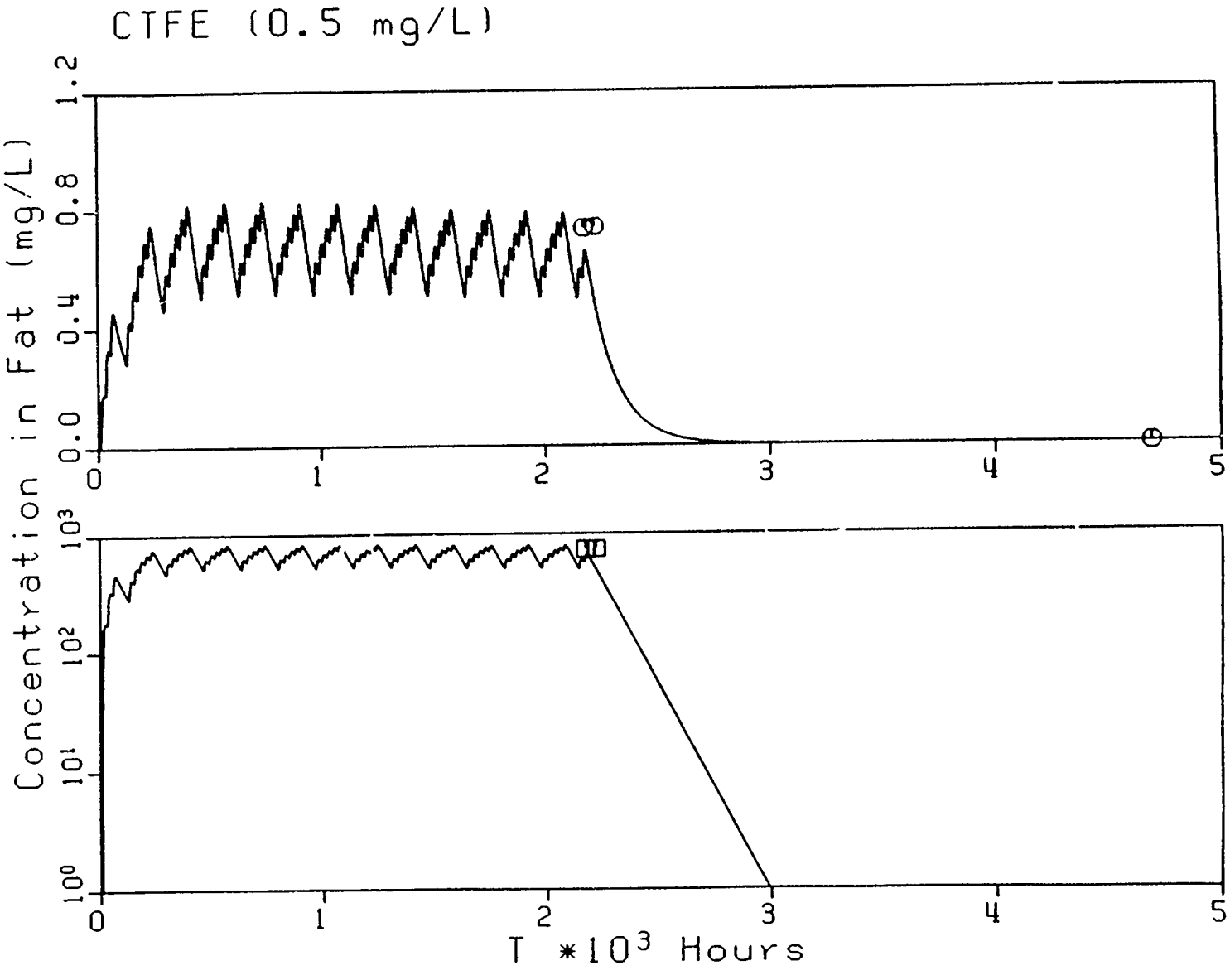

Figure 3.8-9. **Concentration of CTFE (Group I Oligomers) in Fat during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

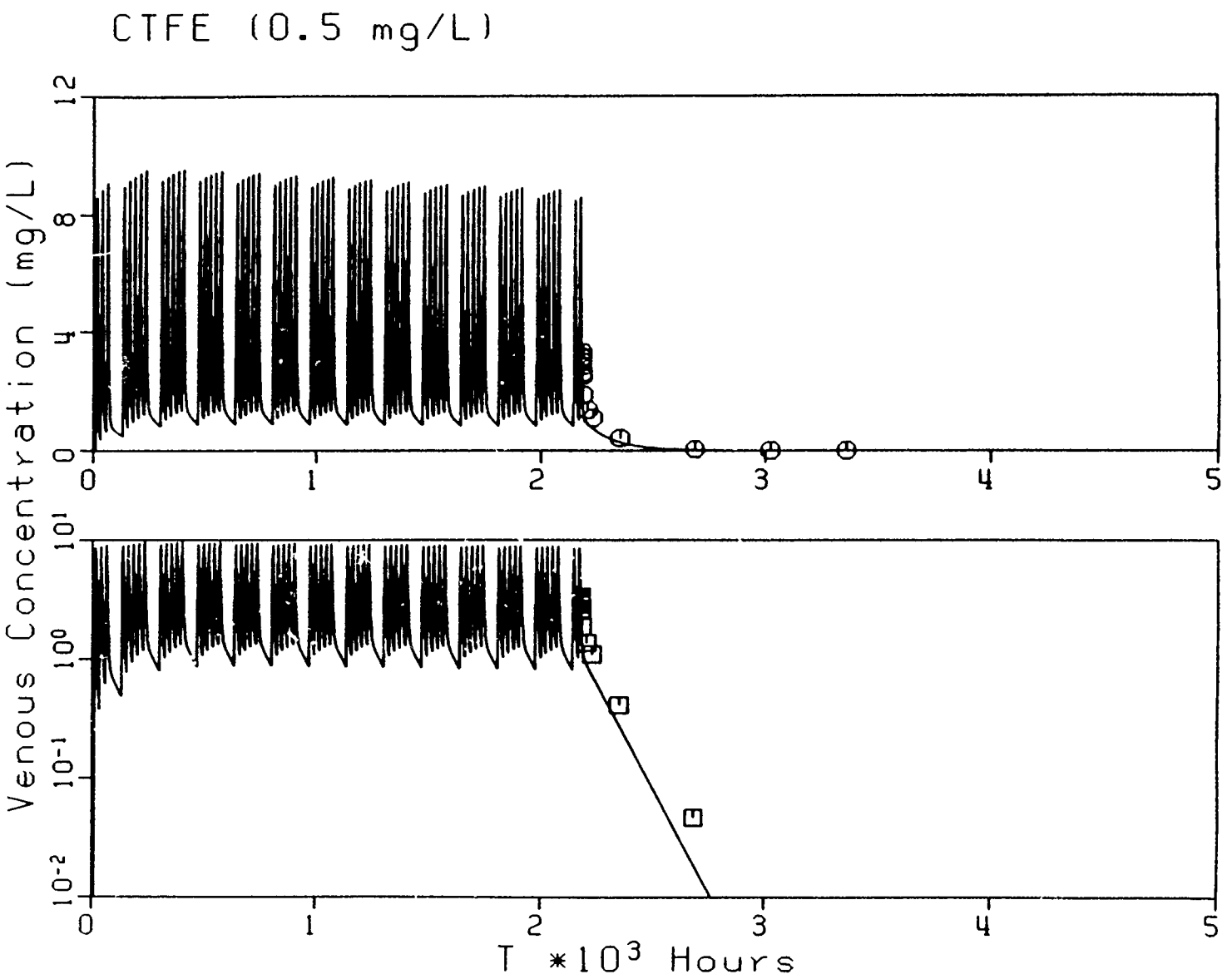

Figure 3.8-10. **Concentration of CTFE (Group I Oligomers) in Venous Blood during and after Single 0.5 mg/l., 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points Log plot, lower curve and square points

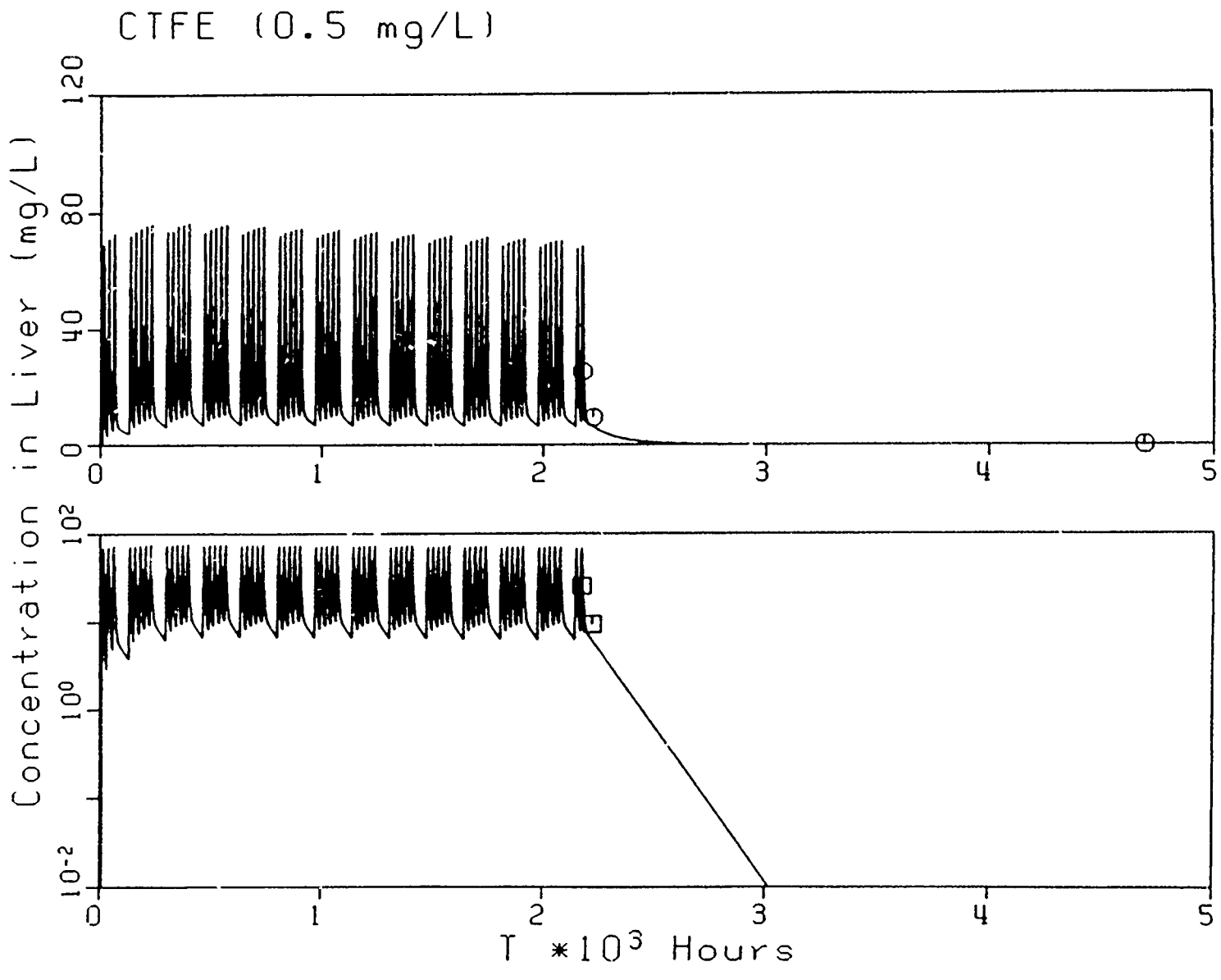

Figure 3.8-11. **Concentration of CTFE (Group I Oligomers) in Liver during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points

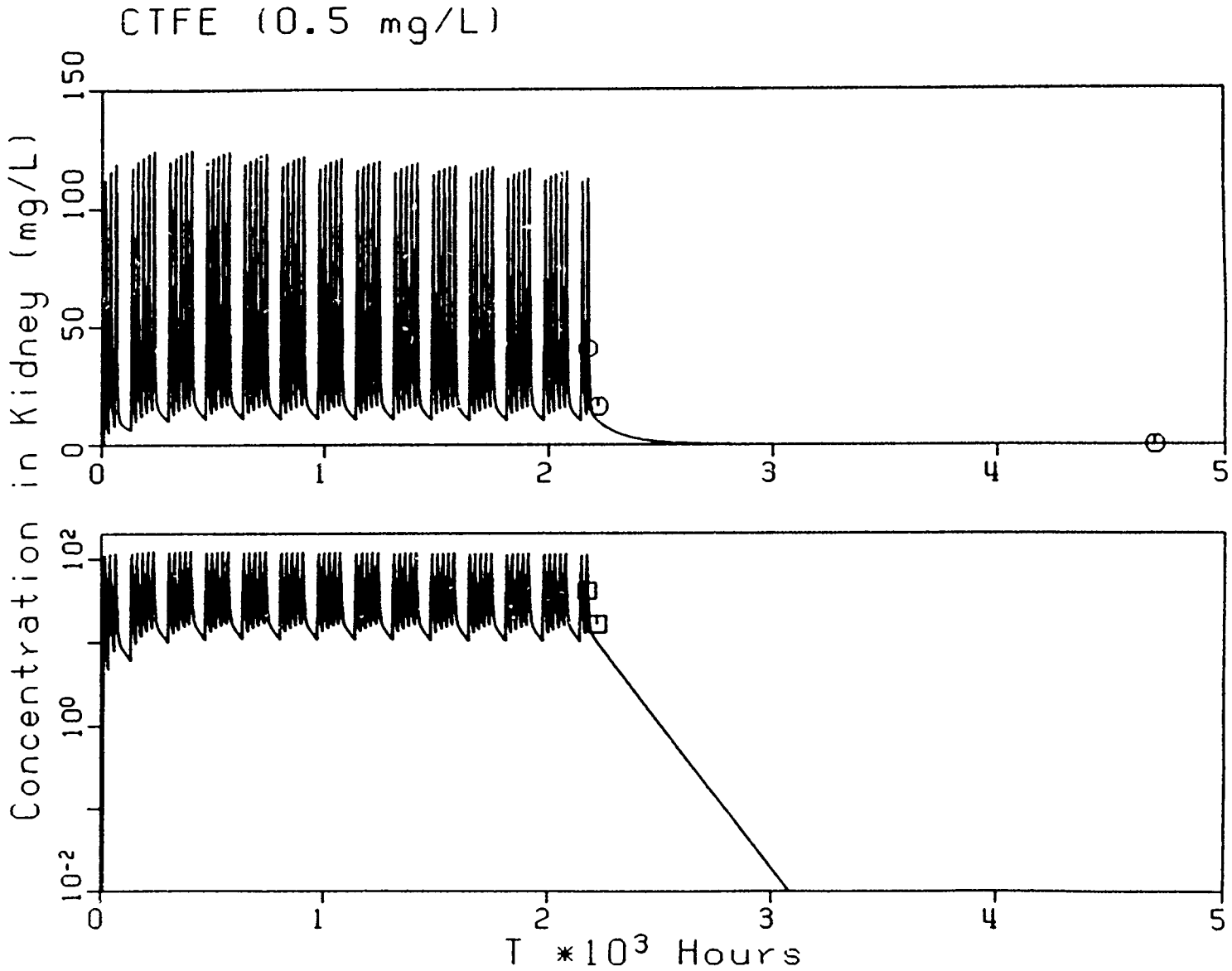

Figure 3.8-12. Concentration of CTFE (Group I Oligomers) in Kidney during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

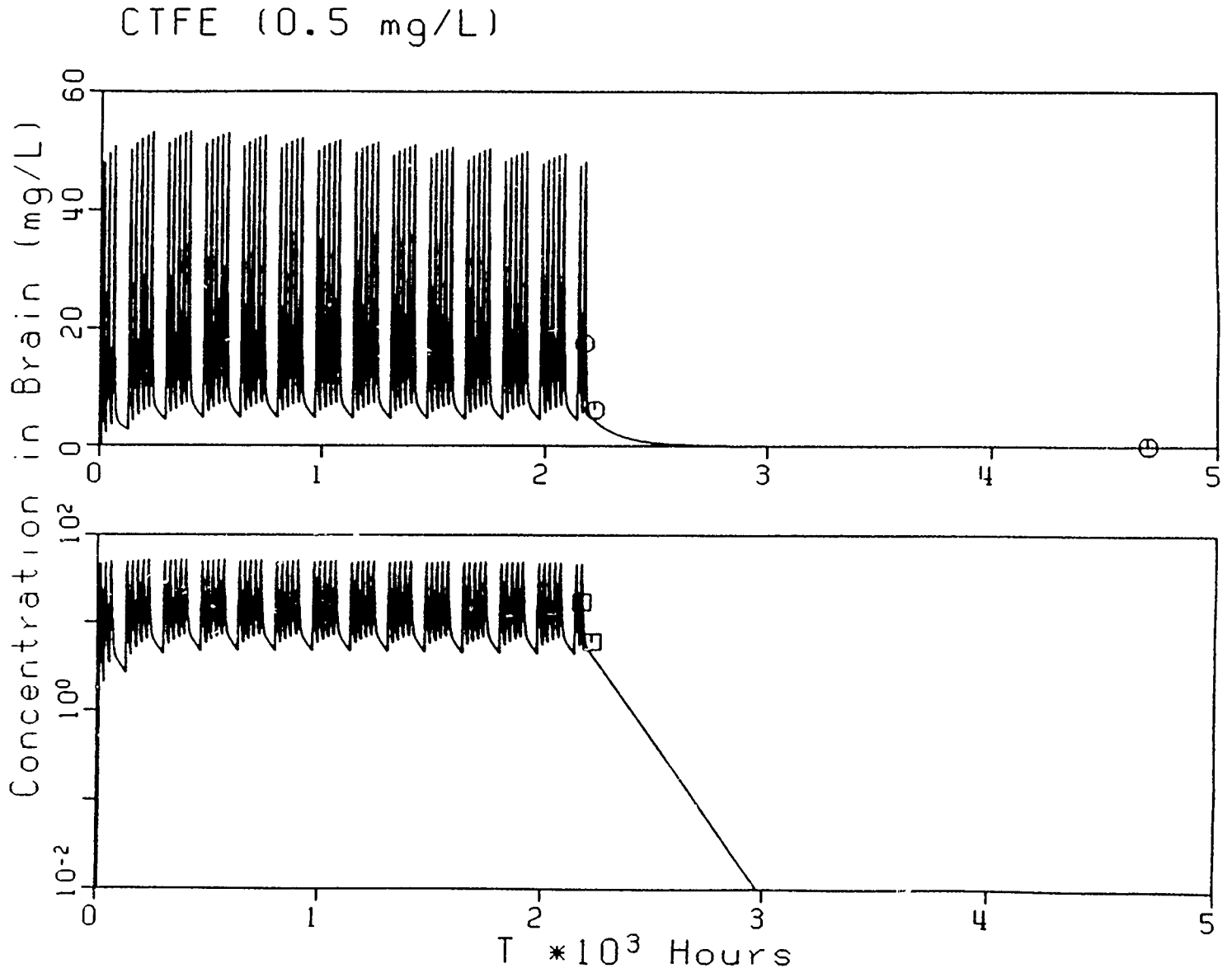

Figure 3.8-13. **Concentration of CTFE (Group I Oligomers) in Brain during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points

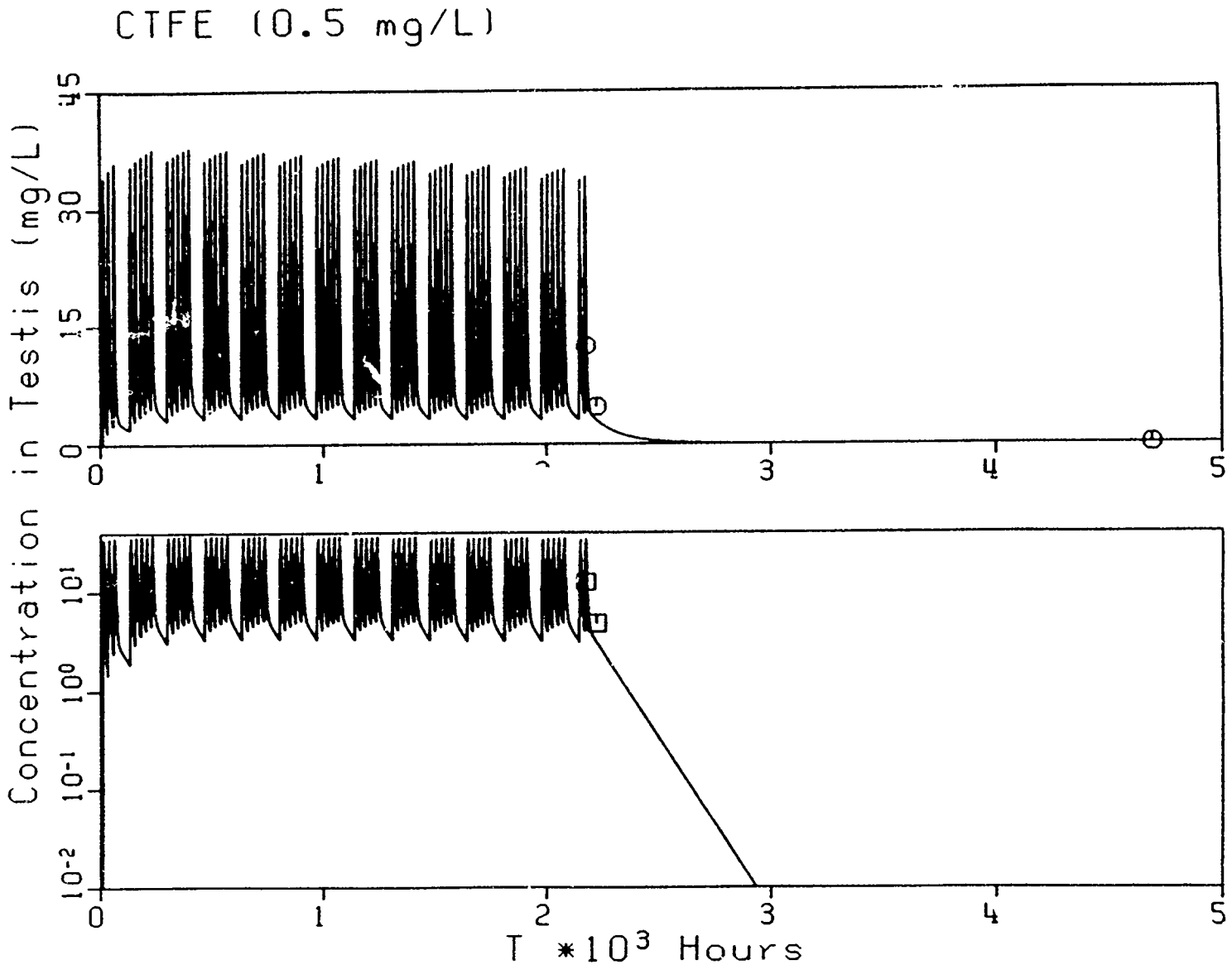

Figure 3.8-14. **Concentration of CTFE (Group I Oligomers) in Testis during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

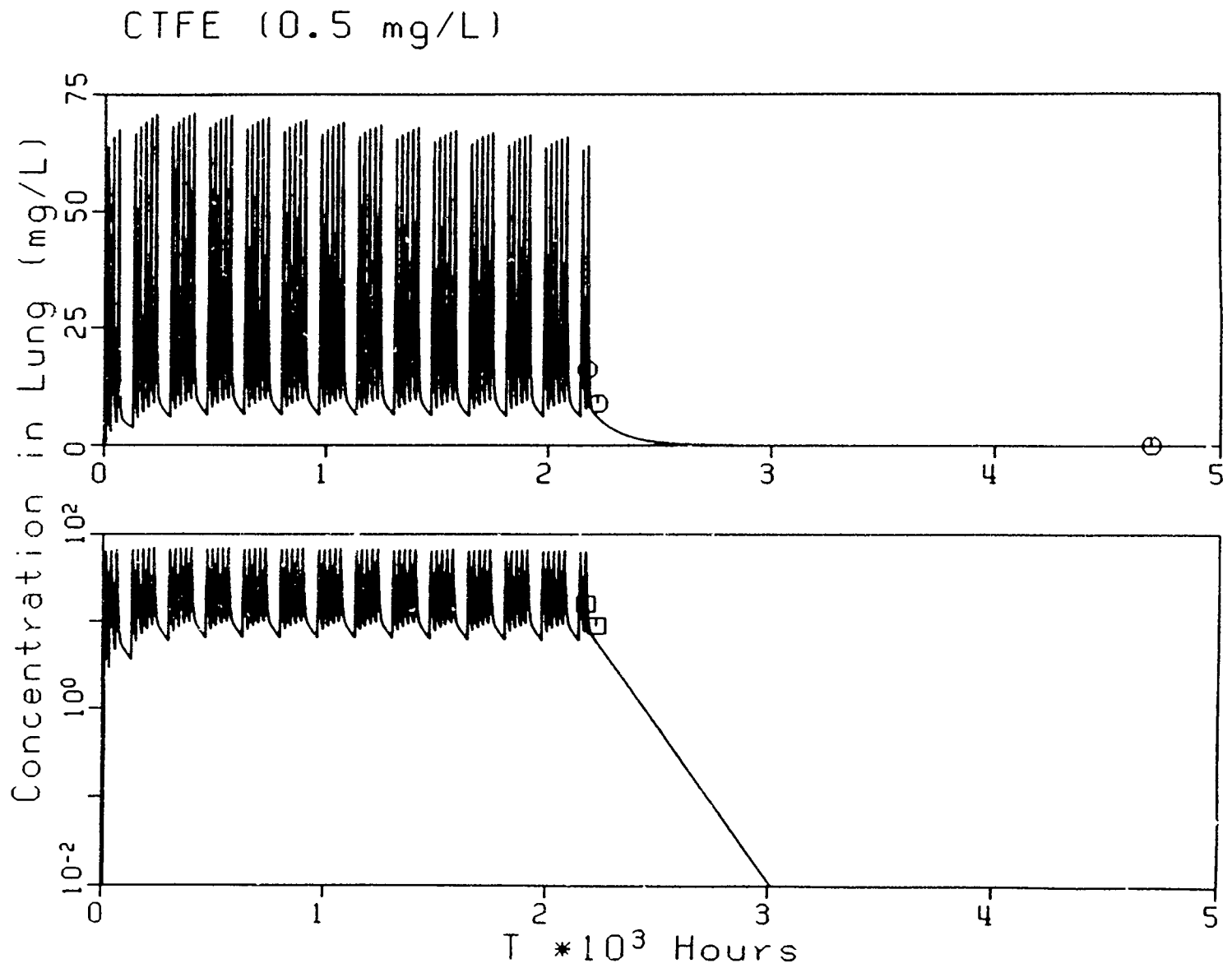

Figure 3.8-15. Concentration of CTFE (Group I Oligomers) in Lung during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure. Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

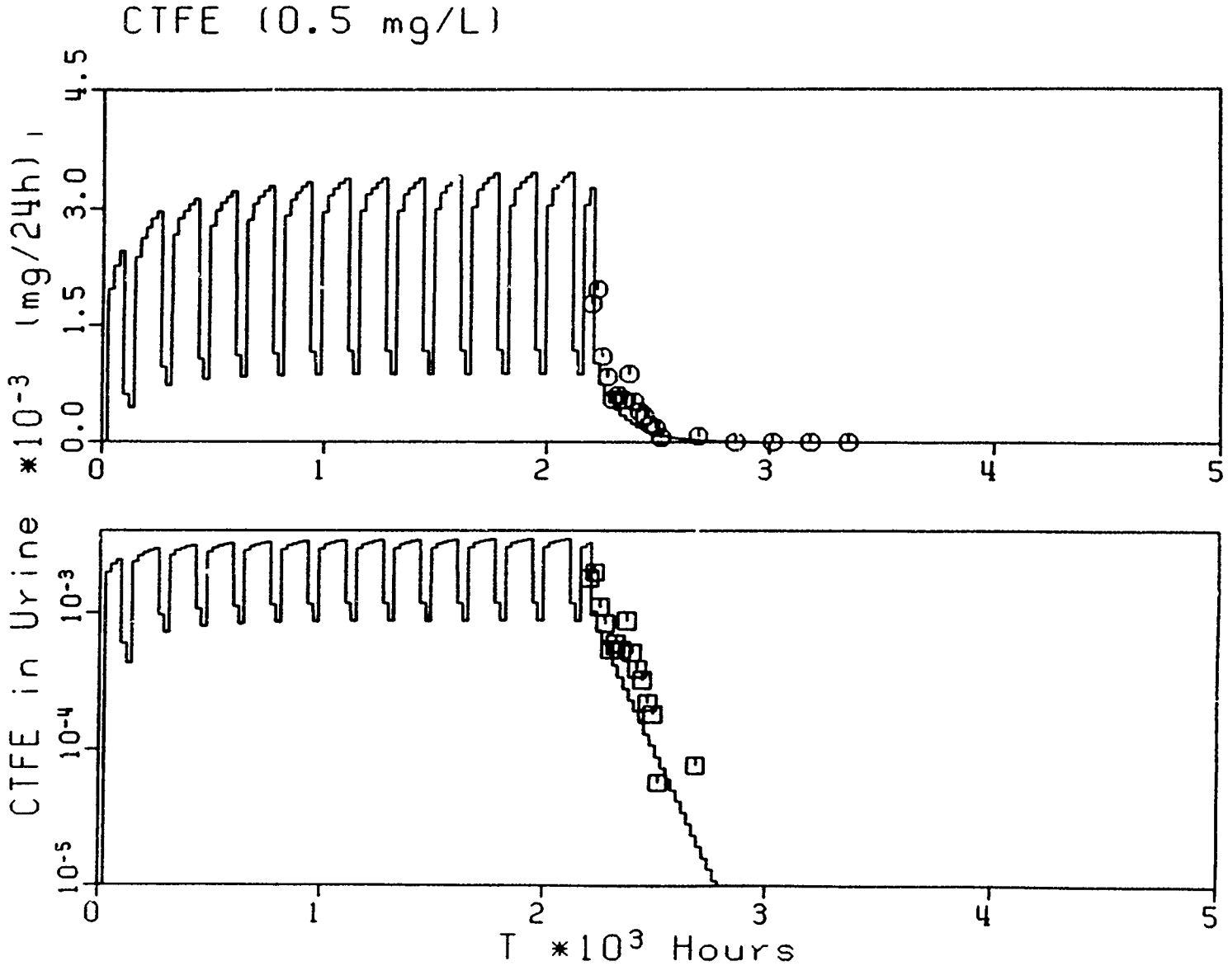

Figure 3.8-16. **CTFE (Group I Oligomers) Excreted in Urine Every 24h during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure.** Solid lines represent simulations, points represent data. Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points

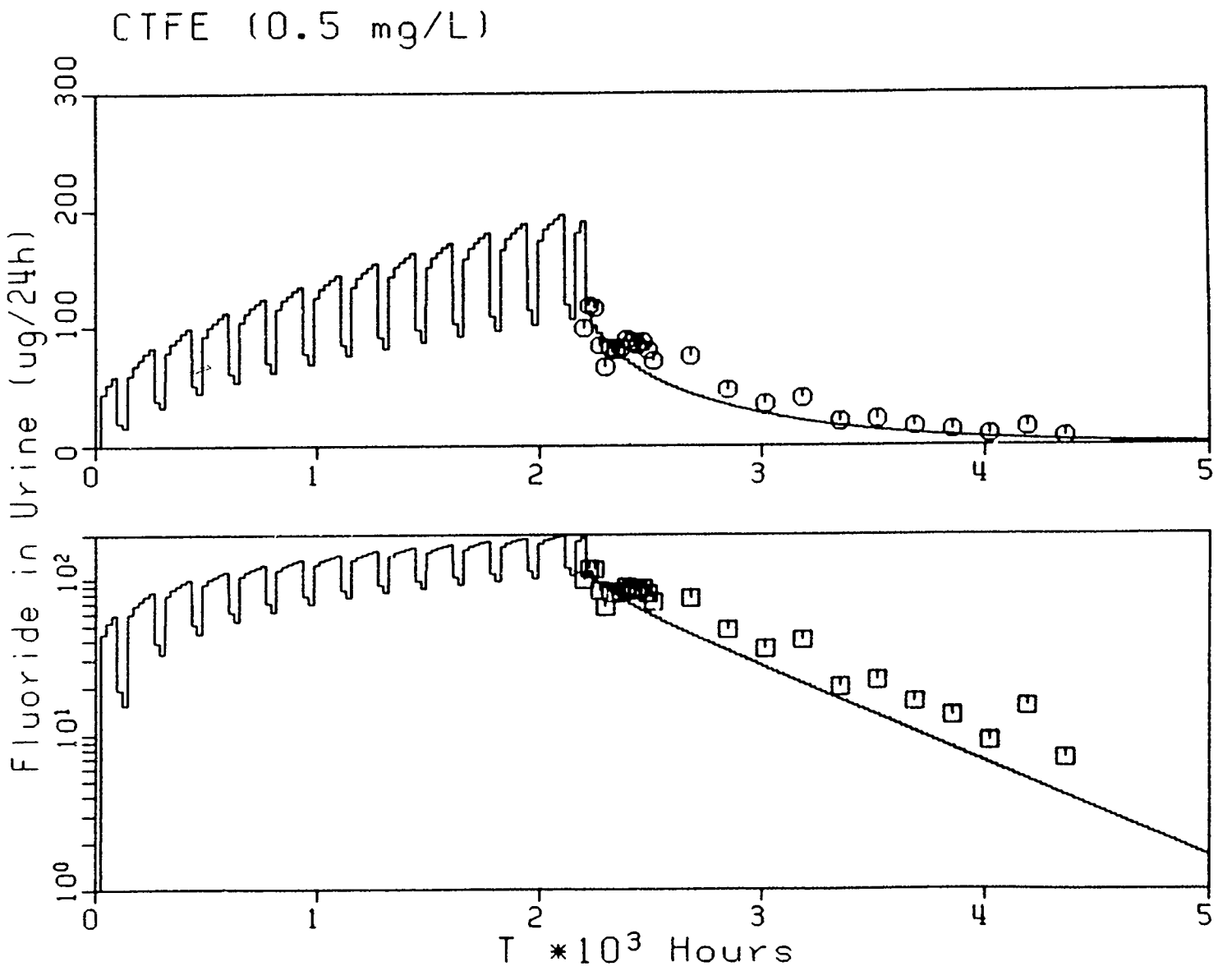

Figure 3.8-17. Amount of Inorganic Fluoride Excreted in Urine Every 24h during and after Single 0.5 mg/L, 6-h/day, 5 days/week, 90-Day Exposure. Solid lines represent simulations, points represent data Linear plot, upper curve and hexagonal points. Log plot, lower curve and square points.

3.9 THE EVALUATION OF THE SENSITIZATION AND ACUTE DERMAL IRRITATION POTENTIAL OF AIR FORCE CANDIDATE AND INVENTORY CLOTHING MATERIALS

E.R. Kinkead, B.T. Culpepper, and S.S. Henry

INTRODUCTION

Four candidate garment fabrics were under consideration by the Air Force for clothing applications in high temperature or fire situations. These materials would be used to replace fiberglass fabrics presently in use that may be irritating to the skin. The studies reported here were designed to evaluate the dermal irritancy and skin sensitization potential of selected candidate fabrics, and to compare the findings to those obtained from present inventory non-fiberglass-containing Air Force garments, as well as to standard solutions of known irritants. These studies were needed to fully evaluate the candidate fabrics for possible use by the Air Force.

The U.S. Environmental Protection Agency's Health Effects Testing Guidelines (1985) recommends the use of young adult guinea pigs for sensitization testing and albino rabbits for skin irritation testing Existing alternative methods to animal testing are inadequate for this study

MATERIALS

Test Agents

Four candidate materials were provided by the Air Force for skin irritation and sensitization testing. They were accompanied by the following identifying information.

1. Gentex, Inc , activated carbon fiber 25%, Nomex Aramid fiber 75%, WPAFB A Sharyn 5556X5 (candidate material "A")

2 Rohm and Haas, Inc., carbonaceous resin combined with PBI fiber, WPAFB B Sharyn 5556X5 (candidate material "B")

3. Winfield, Inc., laminated Nomex shell fabric. Middle layer: VonBlucher carbon spheres. Inner layer: Nomex tricot knit comfort liner fabric (candidate material "C")

4. Celanese, Inc , blue laminated face; Nomex/PBI 20/80. Middle layer: VonBlucher carbon spheres. Inner layer: 100% nylon tricot (black). WPAFB E Sharyn 5556X5 (candidate material "E")

The two laminated fabrics, candidate materials C and E, had the inner layer in contact with the animals' skin during testing

In addition to the candidate materials, the following present inventory, non-fiberglass materials were submitted by the Air Force for comparison testing (skin irritation only).

1. Flight suit (possibly Nomex blend)

2. Trouser, all cotton, fatigue

3. Trouser, Perma Press, fatigue

Solutions of the following five substances were used in the skin irritation and sensitization studies as positive or negative controls

1. Chlorodinitrobenzene (CDNB) (1-chloro-2,4-dinitrobenzene) grade 1, lot #44F-0565, Cat #C-6393, Sigma Chemical Co , CAS #97-00-7 (sensitization control)

2. Carbowax 4000 (polyethylene glycol No. P-3640) powder, lot #95F-0346, Sigma Chemical Co. (sensitization control)

3. Glacial Acetic Acid, Reagent ACS, lot #113, Eastman Kodak Co., CAS #64-29-7 (irritation control)

4. Sodium lauryl sulfate, electrophoresis grade, lot #C15B, Cat #118 1502, Eastman Kodak Co., CAS #151-21-3 (irritation control)

5. Water, laboratory pure (irritation control)

Animals

New Zealand White rabbits, weighing between 2 and 3 kg were purchased for use in the skin irritation study from Clerco Research Farms, Cincinnati, OH. Male, albino, Hartley strain guinea pigs, weighing between 200 and 250 g upon receipt were purchased for use in the sensitization test from Murphy Breeding Laboratory, Inc., Plainfield, IN.

Quality control assessment during a two-week quarantine period confirmed the acceptable health of the proposed study animals The rabbits and guinea pigs were housed individually, the rabbits in wire-bottom stainless-steel cages and the guinea pigs in plastic cages with wood chip bedding Water and feed (Purina Rabbit Chow #5320 and Purina Formulab #5015) were available *ad libitum*. All animals were maintained on a 12-h interval light/dark cycle.

EXPERIMENTAL APPROACH

Skin Irritation

A patch test was utilized to determine the degree of primary skin irritation on intact and abraded skin of albino rabbits In addition to the four candidate materials and three present-inventory materials, three control solutions of moderate, mild, and negative irritancy were included for comparison purposes. The control solutions were as follows. moderate irritant – 5% sodium lauryl sulfate; mild irritant – 30% acetic acid; and non-irritant – laboratory-pure water.

Each test or control material was applied to the skin of six rabbits. To decrease the total number of animals required for this study, two materials were tested concurrently per rabbit

Adjacent areas of untreated skin on each test animal served as a control. All hair was clipped from the backs and sides of the rabbits 24 h prior to exposure to allow for recovery of the skin from any abrasion resulting from the clipping.

Four exposure areas were delineated on each test animal, two of which were abraded by lightly scratching a "#" pattern with a hypodermic needle. The abrasions were minor incisions through the stratum corneum, not deep enough to disturb the derma or produce bleeding Squares of fabric, 0.5 g in mass, were placed on the skin, and 0.5 mL volumes of control solutions were applied to a 3 x 3 cm square area of skin. Each test site was then covered with an approximately 9 cm^2 square of surgical gauze (larger where needed to cover the fabrics), four single layers thick. The gauze and fabric were held in place with strips of surgical adhesive tape, and the entire shaved area was covered with dental dam and secured with an elastic bandage and adhesive tape. To maximize the sensitivity of the irritation test, the samples were left in contact with the skin for 24 h.

After 24 h, the wrap and patches were removed, excess material was wiped from the skin, and each test area was evaluated for irritation using the Draize (1944) scale as a reference Irritation evaluations were conducted also at 48 and 72 h. The total score of the three observations for all rabbits was divided by 18 to yield a primary irritation rating. The primary irritation rating was interpreted using the National Institute for Occupational Safety and Health's skin test rating (Campbell et al., 1975)

Sensitization

The four candidate materials were tested for sensitization potential (present-inventory cloth materials were not included in this study). A positive control material, 0.1% CDNB in acetone, and a negative control, 10% Carbowax 4000 in water, were included for comparative purposes (Horton et al., 1981) using the modified Maguire (1973) sensitization test.

Prior to the actual sensitization testing, a separate group of albino guinea pigs was used to confirm that the test samples were not irritating to guinea pig skin. In addition, 0 1 g of the test fabrics or 0.1 mL of the control solutions were applied to the clipped left flank of the study animals to identify any hypersensitive individuals prior to initiation of the study and remove them from the study group.

For each test sample, the upper backs of 10 guinea pigs were clipped and chemically depilated using Surgex Hair Remover Cream (Sparta Instrument Corporation, Hayward, CA 94545) prior to the first application The shoulder areas of the study animals were then wrapped with a latex bandage to outline the treatment area and to provide a base for holding cover bandages in place. One-tenth gram of fabric or 0.1 mL of control solution was topically applied to the 1.5-cm^2 area, covered by gauze and dental dam, and then secured with adhesive tape This procedure was repeated on

Mondays, Wednesdays, and Fridays until a total of four sensitizing treatments had been applied Along with the third sensitizing treatment, 0.2 mL of a 50% aqueous dilution of Freund's adjuvant (Bacto Adjuvant Complete, Difco Laboratories, Detroit, MI 48232) per animal was injected intradermally using two or three sites next to the test area A two-week induction period followed the removal of the final application

Following the two-week induction period, the left flank was clipped and challenged with 0.1 g or 0.1 mL of test or control material. In order to secure the fabrics in place, they were positioned within a square opening cut from a 2-in -wide latex band wrapped around the pelvic girdle. The fabric was then overlayed with two single layers of surgical gauze and the entire assembly was secured with a band of elastic adhesive tape As a control for erythema or edema resulting from the bandaging, a group of three guinea pigs from the same lot as the study animals were wrapped similarly and used for comparison in scoring the challenge treatment. The challenge applications of the control solutions were not occluded.

All bandages were removed at 24 h, at which time the challenge sites were examined and scored The animals that were wrapped to secure the test fabrics were scored relative to the control wrapped animals; that is, erythema or edema was scored only if greater than that exhibited by the wrapped reference animals. All animals were again examined and scored at 48 h In scoring this test the important statistic is the frequency of the reaction An animal eliciting a score of two or more at 48 h was rated as a positive responder

RESULTS

Skin Irritation

No edema or necrosis was observed in any of the rabbits exposed to the four candidate materials or the three present-inventory materials Only one rabbit exhibited erythema after exposure to these fabrics, and that individual showed only very minor redness on intact (non-abraded) skin at 48 and 72 h after exposure to candidate material E As anticipated, the positive control materials, 5% sodium lauryl sulfate and 30% acetic acid, produced varying degrees of erythema and edema on both intact and abraded skin Primary irritation ratings of all cloth and negative control materials were zero

Sensitization

The guinea pigs were challenged with one of the four candidate materials or one of two control solutions two weeks after the sensitization treatments Scoring at 24 h was complicated by the presence of erythema from the wrappings needed to secure the fabrics in place The erythema was present in both study animals and wrapped controls, however, the irritation was resolved at 48 h.

None of the animals exposed to the candidate materials displayed any signs of sensitivity to the fabrics at 48 h. Eight of 10 guinea pigs treated with the control substance CDNB became sensitized and, as expected, there were no signs of sensitization to the Carbowax 4000 solution.

DISCUSSION

One of six rabbits treated with candidate cloth material E exhibited slight erythema on intact skin at 48 and 72 h. However, no irritation occurred on the adjacent abraded site where a greater response would be expected. None of the remaining five rabbits treated with this cloth material demonstrated an irritative response at any of the evaluation periods. Candidate materials A, B, and C demonstrated no irritation response following 24 h contact.

On the basis of the results of this study, the four candidate cloth materials would not be considered primary skin irritants. Likewise, none of the cloth materials demonstrated a potential for skin sensitization.

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SECTION 4

STUDIES ON INSTALLATION RESTORATION PROGRAM CHEMICALS

4.1 METABOLISM OF TRICHLOROETHYLENE AS MEASURED WITH AN ISOLATED VENTILATED PERFUSED LUNG PREPARATION

A. Vinegar, K.L. Auten, Y.M. Reed, and R.B. Conolly

INTRODUCTION

Physiologically based pharmacokinetic (PB-PK) models have been developed which incorporate quantitative descriptions of toxicant metabolism These descriptions often combine all such metabolic activity in the liver, which is a useful simplification for many cases There are situations, however, where it is desirable to specify non-hepatic biotransformation For example, correlations between toxicant metabolism in non-hepatic target tissues and toxic effects are sometimes needed. With respect to the lung, which is the subject of this report, such correlations are important because of human exposure to volatile environmental and workplace contaminants. Another aspect of pulmonary biotransformation needing evaluation is the extent to which it functions as a first-pass clearance mechanism for inhaled toxicants If pulmonary metabolism does act in this manner it would be analogous to the hepatic first-pass clearance of ingested materials The isolated ventilated perfused lung (IVPL) described in this report and the data generated with it will contribute to an understanding of these problems.

The nonrespiratory metabolic functions of the lung have received increased attention in recent years. Knowledge of these functions and interest in the fate of xenobiotic substances have resulted in increased use of IVPL preparations as an attempt to estimate the lungs' contribution to the metabolism of these substances In some cases, the lungs themselves may be the target for toxic effects of either the parent compound and/or its metabolites.

There is not an adequate technique for studying pulmonary metabolic activity *in vivo* because of the influence of other organs Sampling pulmonary arterial and venous blood to determine concentrations of chemicals *in vivo* is of questionable value (Rochester et al , 1973, Tierney, 1974) The IVPL preparation maintains relatively "normal" anatomical and physiological relationships between the lung and its circulation The IVPL also provides the opportunity to study effects of agents administered into either the pulmonary perfusate or the inhaled air supply Recent reviews of the use of IVPL preparations, with discussion of methods and of compounds studied, have been prepared by Mehendale et al (1981) and Niemeier (1984) Results obtained from metabolic studies using the IVPL will serve as input data to PB-PK models where the compartments of the model are discrete organ

systems with associated blood flows, organ volumes, and partition coefficients (Ramsey and Andersen, 1984).

The studies described in this report were conducted to quantitate pulmonary metabolism of trichloroethylene (TCE), a chemical of interest because of its frequent identification in Installation Restoration Program (IRP) sites. TCE has been found as a groundwater contaminant and is a widely used industrial solvent. TCE is used primarily in metal degreasing which consumes about 90% of the annual 130,000 metric tons produced in the United States annually. Other uses include dry cleaning, fumigation, and textile processing.

Long-term toxic effects of TCE exposure include liver and kidney injury. Mutagenicity data are conflicting but TCE is carcinogenic in rodents (Agency for Toxic Substances and Disease Registry, 1988). The toxicological effects of TCE are reviewed also in the Agency for Toxic Substances and Disease Registry's publication (1988).

As an aid to conducting these studies, a PB-PK model of the IVPL was developed. Discussion is presented on the challenges encountered quantitating the metabolism of TCE during the conduct of these studies.

MATERIALS AND METHODS

IVPL Set Up

Blood procurement. Male Fischer 344 (F-344) rats that weighed 210-440g were anesthetized with sodium pentobarbital (35 mg/kg). Blood was collected from the dorsal aorta with a heparinized syringe after opening the abdomen. Blood was pooled from several animals to obtain a volume of 25 mL for the perfusion system and several milliliters extra to allow replacement when samples were taken for analysis.

Artificial perfusate. Some experiments were conducted with artificial perfusate instead of blood. The perfusate used was Krebs-Ringer solution. It contained: 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ (anhydrous), and 25 mM $NaHCO_3$ in distilled water and stored at 4°C until needed. Prior to use 4.5% (w/v) dextran and 0.09% (w/v) glucose was added and the solution was titrated with 0.1 N HCl to pH 7.4.

Lung cannulation. Male F-344 rats were anesthetized with sodium pentobarbital (35 mg/kg) Cannulae were placed in the pulmonary artery and trachea. The lungs were attached by these cannulae to the perfusion apparatus.

IVPL apparatus. The IVPL apparatus was similar to that described by Dalbey and Bingham (1978). Major differences were that the blood reservoir was closed to the outside atmosphere

preventing the loss of volatile compounds from the system and a blood flow probe was used to measure flow through the system

Briefly, the system (Figure 4.1-1) consisted of a perfusate reservoir, in which the perfusate was kept at a constant level of 23 cm above the hilum of the lung The reservoir emptied into the top of the artificial thorax via a tube to which the arterial cannula of the lung was attached. Venous outflow of the lung dripped to the bottom of the thorax and was pumped back (Masterflex Tubing Pump, Cole Palmer, Chicago, IL) into the reservoir at a constant rate. Perfusate flow was monitored (Square-Wave Electromagnetic Flowmeter, Carolina Medical Electronics, Inc., King, NC) so that pump speed could be adjusted to maintain constant flow The tracheal cannula of the lung was connected to a tube that lead outside the thorax Thus, by way of a t-shaped connection either fresh air or test compound could be presented to the breathing zone of the lung A third tube connected the thorax to a respiratory ventilator (Model 680, Harvard Apparatus, South Natick, MA) through a negative pressure source, allowing negative pressure swings in the thorax to ventilate the lung.

Computer Simulation Model of IVPL

A structural model of the IVPL was written in Advanced Continuous Simulation Language (ACSL) Besides the lung itself, the whole perfusion circuitry as described above was included in the model Each section of the apparatus represented a compartment and thus had a volume and a set of equations to describe the mass flow of parent compound and its metabolites. The general forms of the equations follow those described by Ramsey and Andersen (1984) Use of the model is illustrated in the results section.

Methods for Analysis of TCE and Its Metabolites

TCE is metabolized in the rat lung to trichloroethanol (TCOH) and trichloroacetic acid (TCA) (Dalbey and Bingham, 1978). Analyses were conducted of the inhalation exposure concentrations of TCE and of the perfusate concentrations of TCOH and TCA from samples taken every 10 to 20 min

TCE – One-half milliliter vapor samples were removed using a gas-tight syringe and injected into the gas chromatograph (GC) for analysis. GC calibration was accomplished with standard bags.

TCOH – Fifty microliter of perfusate was mixed with 1 mL N-hexane in a 1.5-mL septum vial and then incubated in a 37°C incubator/shaker for 1 h One µl of the hexane layer was injected into a GC for analysis Measured amounts of TCOH in hexane were used as standards Standards were not made up in blood because of the logistics of blood collection Hexane extraction of TCOH from blood was about 90% efficient

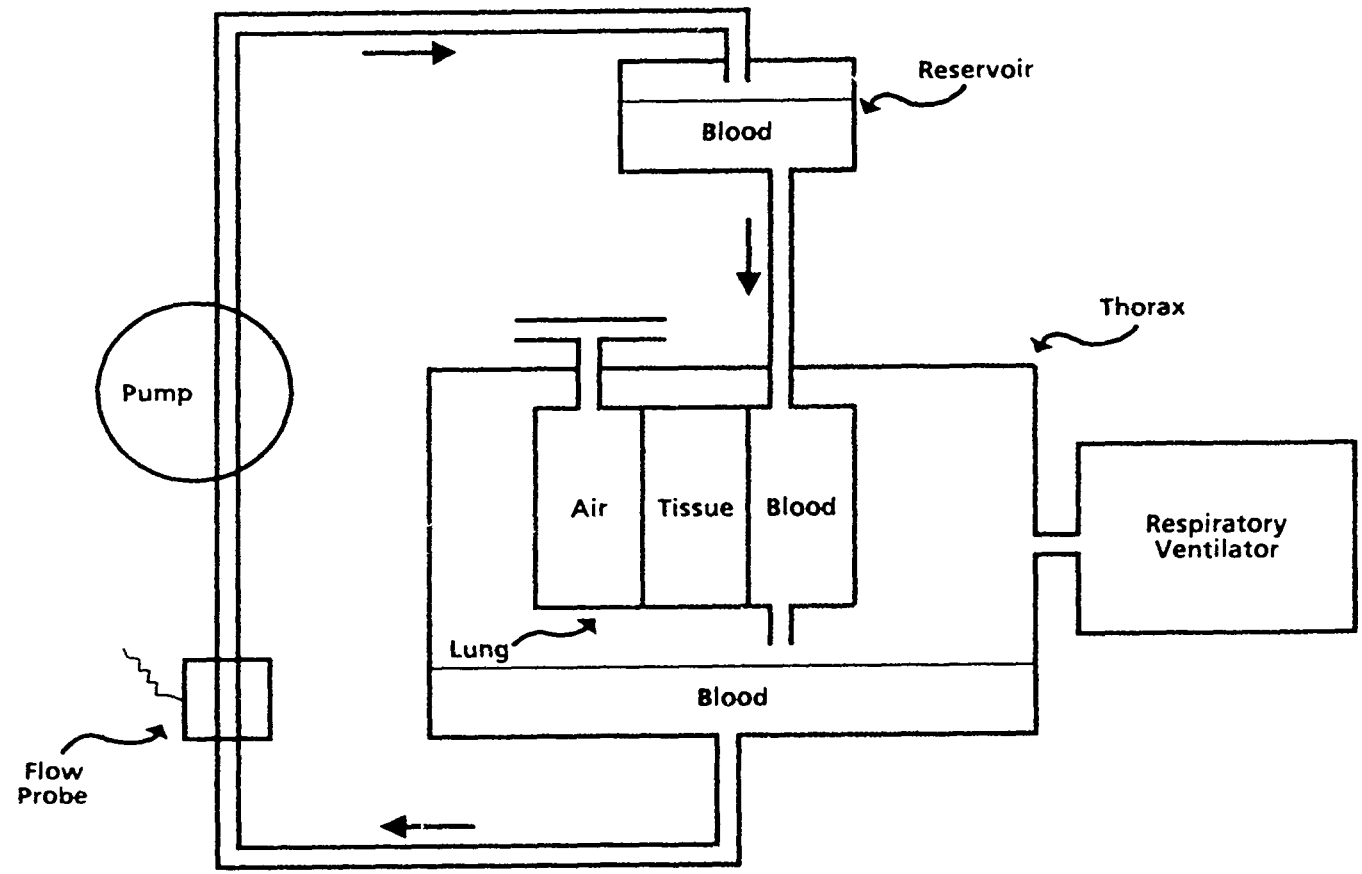

Figure 4.1-1. **Block Diagram of Major Components of the IVPL Preparation.** Arrows indicate the direction of blood flow through the system The lung is shown schematically and is separated into the three component parts considered in the PB-PK model

TCA – Perfusate samples were collected and centrifuged, yielding serum. Fifty microliters of serum were added to 100 µL methanolic HCl and mixed with 1 mL N-hexane. Samples were incubated at 100°C for 30 min. One microliter of the hexane layer was injected into a GC for analysis For standards, measured amounts of TCA were made up in distilled H_2O and run through the same procedure as the samples.

GC Conditions – TCOH and TCA were detected using Hewlett-Packard (Model 5890A) GC equipped with an electron capture detector with a 12 ft x 1/8 in. stainless-steel column packed with 10% SE-30 on 80/100 Chromosorb. TCE was detected using a flame ionization detector with a 6 ft x 1/8 in. stainless-steel column packed with 0 1% SP-1000 on 80/100 Carbopack C.

RESULTS

The major purpose of studying the metabolism of TCE by the lung was to determine pulmonary metabolic constants, V_{maxl} and K_m, for use in a whole-body PB-PK model of TCE. The PB-PK model developed for the IVPL was used as an aide for determining V_{maxl}, i e., the maximum rate of metabolism by the lung. Whole body V_{max} for TCE was set at 11 0 mg/h/kg of body weight (Andersen et al., 1987). Lung metabolism was then expressed as a fraction of whole body metabolism.

Metabolism was measured initially with the lung exposed to 200 ppm of TCE and with blood as the perfusate This exposure concentration was selected based on literature values that indicate that the pulmonary K_m for TCE was well below this level (Dalbey and Bingham, 1978) Exposures to 400 ppm were then conducted to assure saturable conditions for lung metabolism of TCE had been attained at 200 ppm. Exposures to 200 and 50 ppm were conducted using artificial perfusate to determine the efficacy of its use, instead of blood, in future experiments. It was possible to compare data obtained with artificial perfusate to those obtained with blood because of the simulation approach and knowledge of partition coefficients (perfusate air partition coefficient 1 075 compared to 21 9 for blood) Exposure to 200 and 50 ppm with artificial perfusate produced similar perfusate concentrations of TCE as exposure to 9 82 and 2 45 ppm with blood

Appearance of TCOH was shown during exposure to 400 and 200 ppm TCE with blood (Figures 4 1-2 and 4.1-3) and to 200 and 50 ppm of TCE with artificial perfusate (Figures 4 1-4 and 4 1-5) The slopes of the lines coplotted with the data represent the simulated accumulation of TCOH produced by the lung Delays, seen in the first appearance of metabolite, are not accounted for in the model, which predicts a constant rate of appearance from zero time The delay could be due to diffusional limitation on the movement of TCE into and TCOH out of the lung As no apparent increase in rate of metabolism above 200 ppm was seen (compare Figures 4 1-2 and 4 1-3), lung metabolism as a fraction of whole body metabolism was fitted so that the simulated rate of TCOH production matched the actual data points for the 400 and 200 ppm exposures Then, using data from the 200 and 50 ppm exposures that used artificial perfusate, where metabolism occurred at a

rate lower than V_{maxl}, the slope of the simulation was found to still fit the slope of the data. This indicated that the K_m for the lung and for whole body metabolism of TCE were similar. V_{maxl} and K_m values were estimated to be 0.015 mg/h/kg body weight and 0.25 mg/L, respectively. This V_{maxl} was 0.00135 of whole body V_{max}.

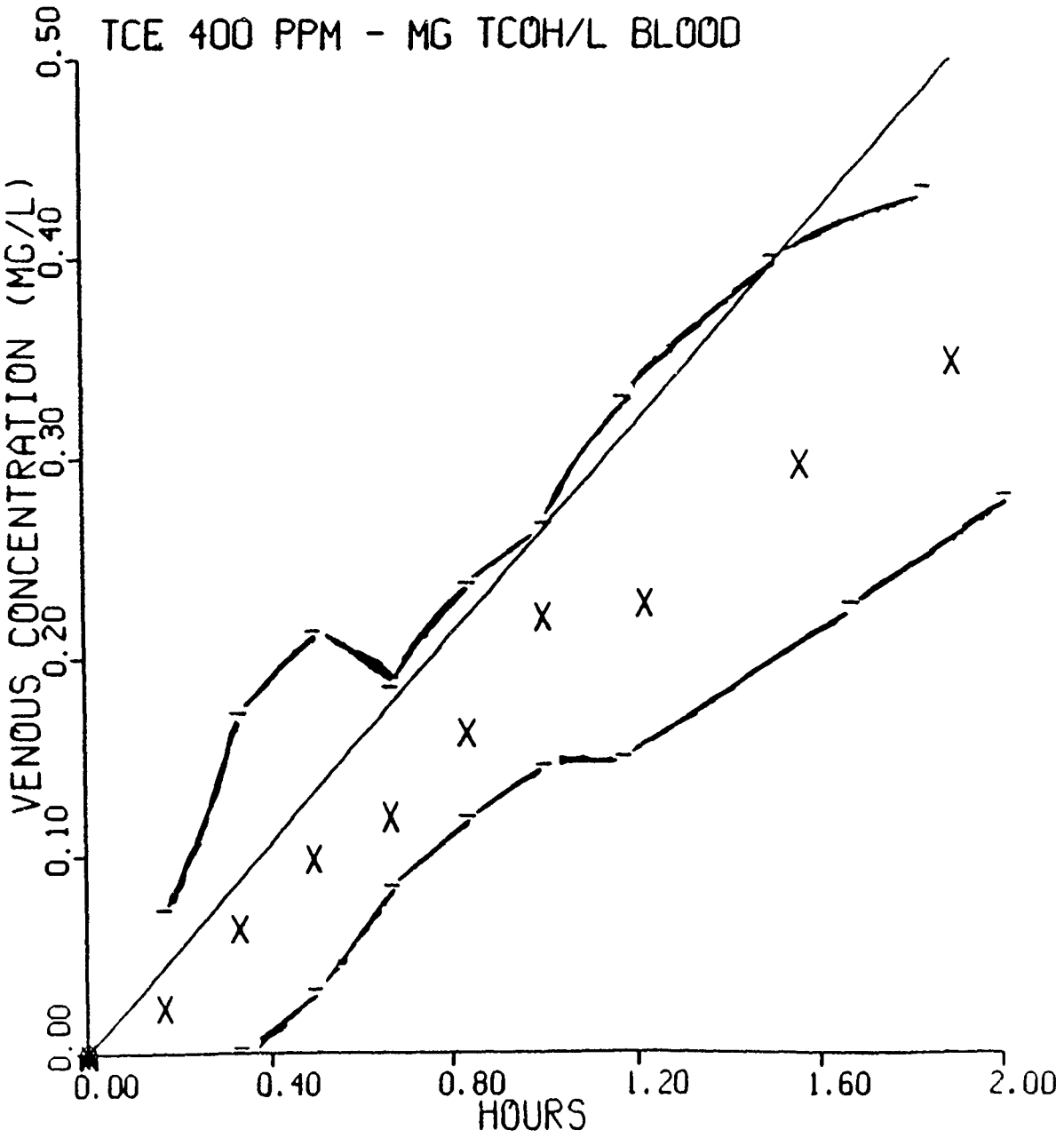

Figure 4.1-2. Trichloroethanol Concentration in Blood Perfusate during Continuous Exposure to 400 ppm of Trichloroethylene. Continuous line represents the simulation; individual points represent actual data. 'X' indicates mean; '-' indicates range.

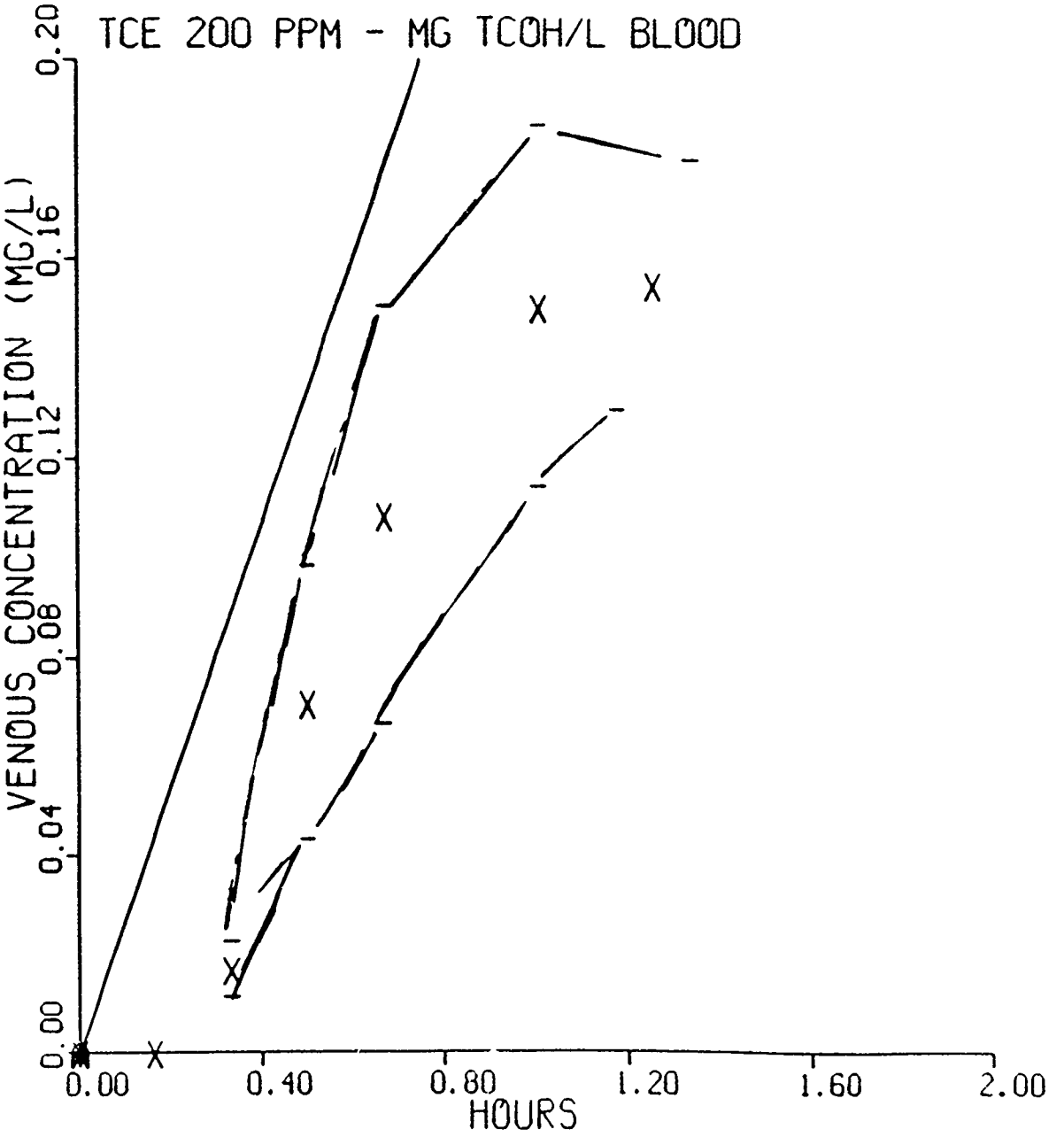

Figure 4.1-3. Trichloroethanol Concentration in Blood Perfusate during Continuous Exposure to 200 ppm of Trichloroethylene. Continuous line represents the simulation; individual points represent actual data. 'X' indicates mean; '–' indicates range.

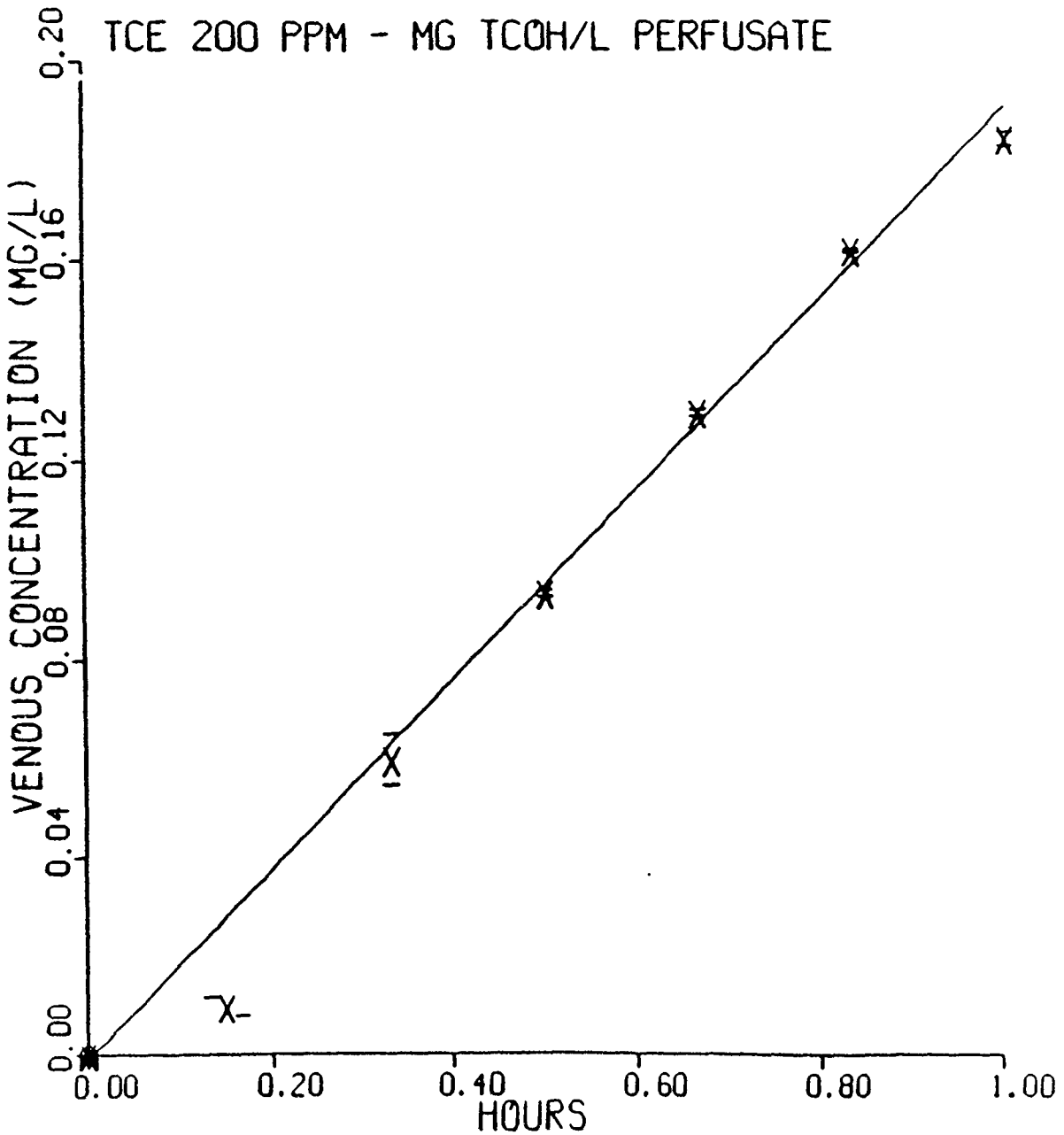

Figure 4.1-4. Trichloroethanol Concentration in Artificial Perfusate during Continuous Exposure to 200 ppm of Trichloroethylene. Continuous line represents the simulation; individual points represent actual data. 'X' indicates mean; '–' indicates range.

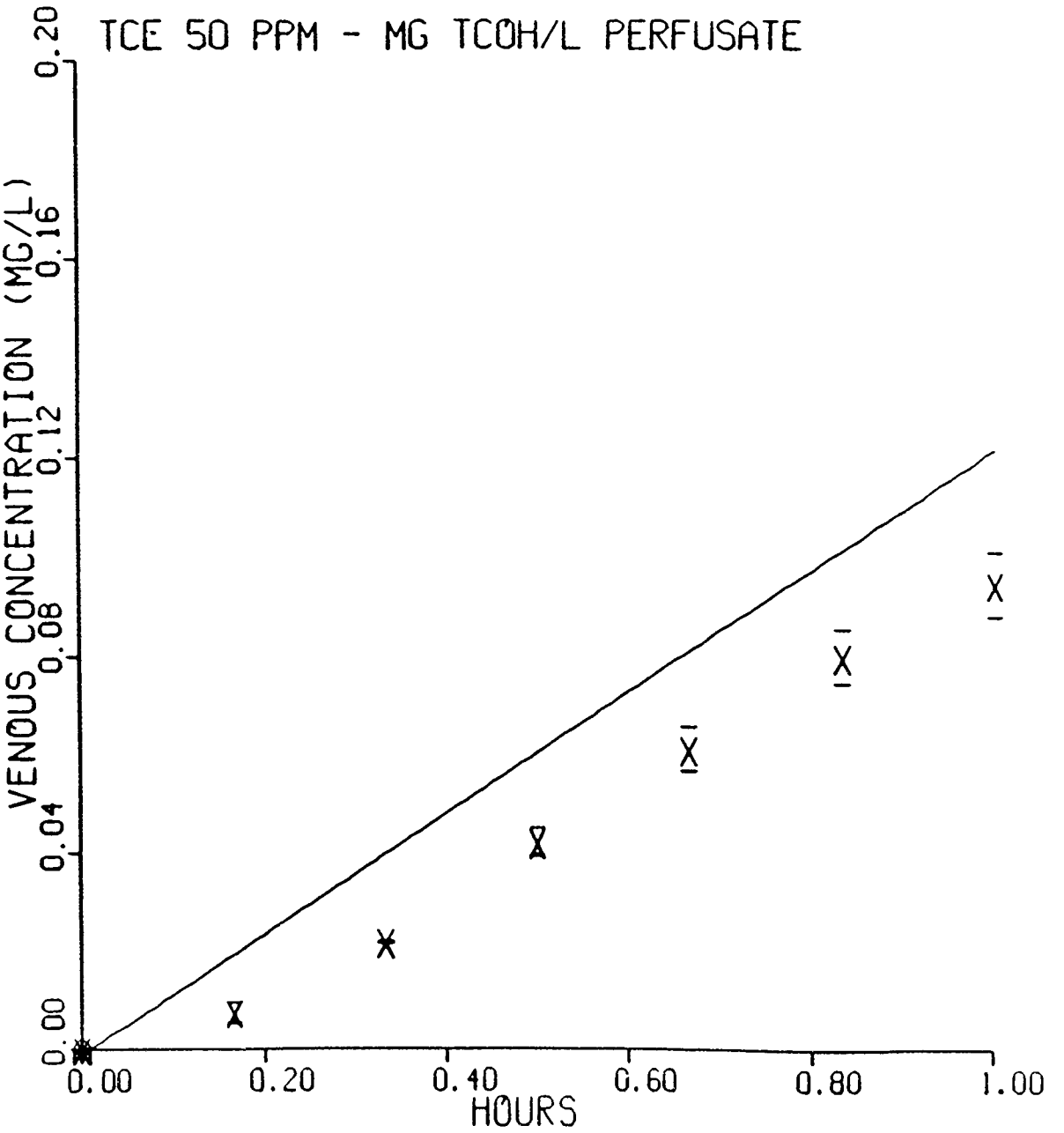

Figure 4.1-5. Trichloroethanol Concentration in Artificial Perfusate during Continuous Exposure to 50 ppm of Trichloroethylene. Continuous line represents the simulation; individual points represent actual data. 'X' indicates mean; '–' indicates range.

No TCA was detected in the perfusate after TCE exposures as high as 400 ppm It was still possible, however, to estimate the pulmonary V_{max} and K_m of TCE. For the modeling of TCOH production described above, lung metabolism of TCE was assumed to result in the production of TCOH and TCA in a three-to-one ratio (Dalbey and Bingham, 1978). However, if all metabolite produced were TCOH the V_{maxl} would be 0.011 mg/h/kg and if there were equal amounts of TCOH and TCA the V_{maxl} would be 0.022 mg/h/kg. These represent rates of lung metabolism at 0.001 and 0.002, respectively, of whole-body V_{max} and probable bounds on the contribution of the lung to the total metabolic capacity of the male Fischer 344 rat for TCE.

Once V_{maxl} and K_m were estimated the model was used to generate concentration response curves for blood and artificial perfusate (Figure 4.1-6). A substantially lower rate of metabolism for a given concentration of TCE was predicted for artificial perfusate than for blood. This was due to the difference in perfusate:air partition coefficients for blood and artificial perfusate . These were 21.9 and 1.075, respectively. This result illustrates that if realistic estimates of pulmonary metabolic capability are to be obtained, it is important to either maintain physiological conditions in the IVPL or, if physiological conditions are not maintained, to have quantitative information on how the system in use differs from the physiologically realistic system. However, the functional equivalence of lungs with the two perfusates still remains a question.

DISCUSSION

Most analytical methods have been developed with sensitivities that are appropriate to concentrations of metabolites encountered as the result of whole body metabolism. The concentrations of TCE metabolites encountered in these studies were at least three orders of magnitude below those obtained from studies of *in vivo* metabolism. Thus, availability of analytical methodology can be an impediment to quantitation of metabolites in the IVPL. The concentrations of metabolite produced were only detectable because the perfusate was recirculated allowing accumulation over time If the efficiency of the lung as a first pass organ for TCE metabolism were to be determined experimentally, it would be necessary to measure metabolite in perfusate that passed only once through the lung which would be impractical experimentally. However, the first pass efficiency of the lung was examined by use of the computer simulation model developed for the IVPL system. The model was altered to simulate an IVPL system configured for a single pass of perfusate through the lung The V_{max} and K_m determined experimentally were used First pass efficiency was expressed in the model as 100 x [TCOH]/([TCE] + [TCOH]) (concentrations in artificial perfusate) The efficiency was less than 0 4%, even at parts-per-billion exposure concentrations, indicating that the lung is a poor first pass organ for TCE metabolism .

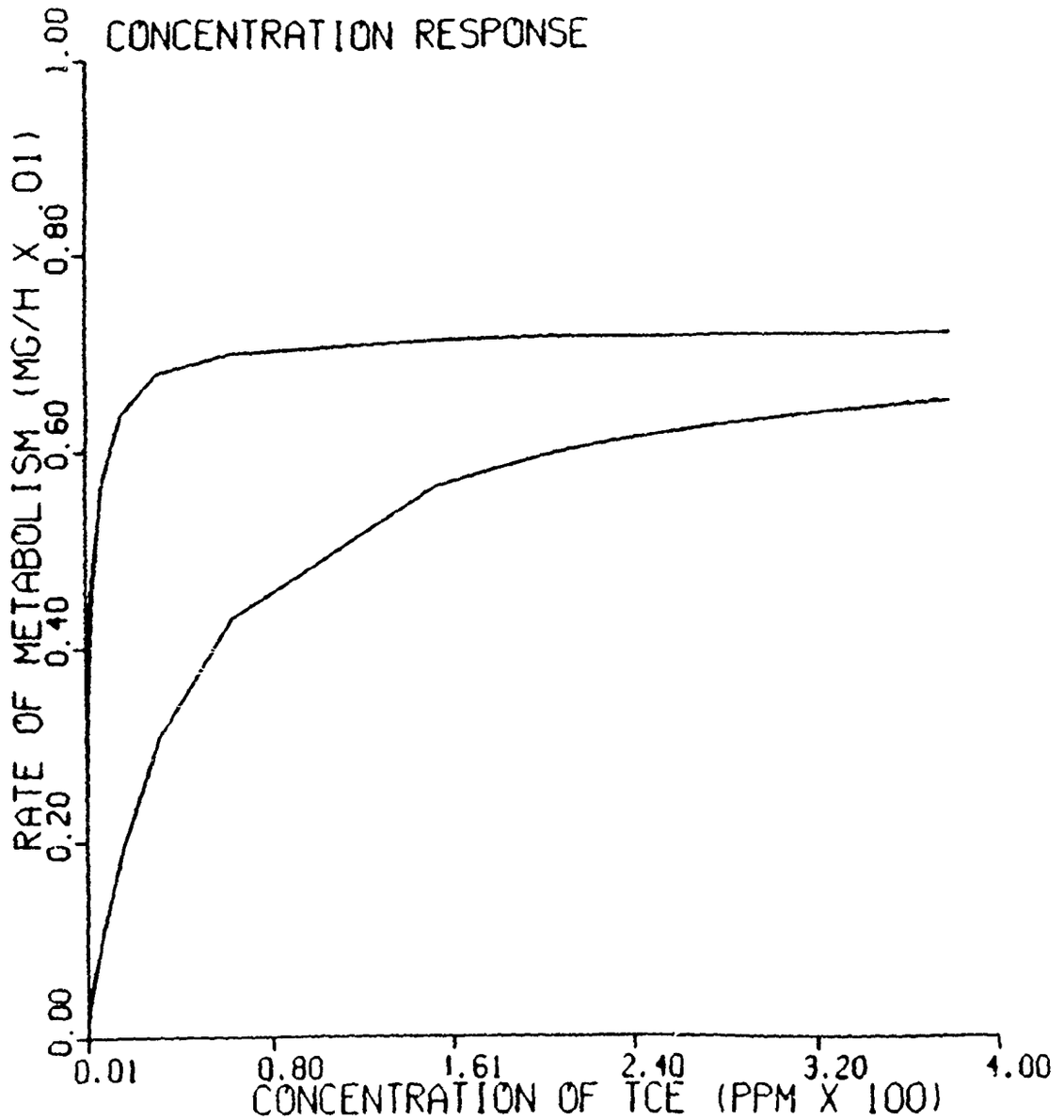

Figure 4.1-6. Simulation Dose Response Relationship for the Pulmonary Metabolism of Trichloroethylene to Trichloroethanol. *Blood* was the perfusate (blood:air partition coefficient of 21.9) for upper curve and artificial perfusate (perfusate:air partition coefficient of 1 075) for lower curve

Investigators using IVPL preparations have continually moved away from the use of blood as the perfusate (Rhoades, 1984). There have always been issues regarding the undefined composition and the state of the donors (dietary, hormonal, etc.) The presence of vasoactive substances (histamine, serotonin) could cause vasoconstriction which would increase vascular resistance (Daley, 1938) and the platelets, damaged during the mechanical pumping, would release serotonin (Norman, 1968). Thus, artificial perfusates have been used more frequently because of their uniformity of composition. Other benefits of artificial perfusates are that fewer animals are needed, less time is needed for setup of a preparation, and thus several experiments can be performed in a day A disadvantage of artificial perfusate with respect to a "physiological" IVPL, however, is the non-physiological perfusate:air partition coefficient The lung/artificial perfusate partition coefficients for the parent compound and its metabolites would have to be the same as those for lung/blood in order to maintain the same concentration relationships between the lung and perfusate in the IVPL as *in vivo* Future studies should address the possibility that an artificial perfusate could be "doped" to give it a physiological partition coefficient.

Measurable concentrations of metabolites at low exposure concentrations could be realized if the size of the blood reservoir, the size of the artificial thorax, and the tubing volume connecting the two were reduced. This tubing passed through the pump head and a pump head that used less tubing would be needed. These changes would reduce the volume of blood in the IVPL preparation and thus the dilution of metabolites. Such changes would probably not allow about a 30% decrease in blood volume. However, this might be enough to bring some metabolite concentrations into the detectable range.

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SECTION 5

STUDIES ON AIR FORCE FUELS

5.1 *IN VITRO* TOXICITY OF SOLUBILIZED 2,3,4-TRIMETHYLPENTANE

N.J. DelRaso, D.R. Mattie[a], C.S. Godin

INTRODUCTION

Trimethylpentane (TMP) is an important determinant of "octane rating" in gasoline. A single gavage dose of 2,3,4-TMP was found to induce a hydrocarbon nephropathy in male Fischer 344 rats This nephropathy was characterized by an increase in cytoplasmic hyaline droplets in the epithelial cells of the proximal tubule. α 2μ-Globulin is believed to be the major constituent of the cytoplasmic hyaline droplets seen in epithelial cells of the proximal tubule of male rats exposed to hydrocarbon vapors (Alden et al., 1985). α 2μ-Globulin is a low molecular weight (MW 26,400) protein produced in the liver of male rats (Irwin et al., 1971) This protein is filtered by the kidney through the glomeruli and is reported to be the major urinary protein in male rats. α 2μ-Globulin is not synthesized by the liver of normal female rats or other species (Roy, 1973).

Recently, Charbonneau et al. (1987) have shown that the concentration of radiolabeled-2,2,4-TMP-derived activity in the kidneys of male rats was 2 4 times higher than that seen in the kidneys of females This increased retention of radiolabeled-TMP-derived act vity was associated with a significant increase in the renal concentration of the male rat-specific α 2μ-globulin protein. These authors also found that the increased 2,2,4-TMP-derived radiolabel in the kidney was attributed to retention of one of the metabolites (2,2,4-trimethyl-2-pentanol) These data suggest that the renal accumulation of the pentanol metabolite and α 2μ-globulin may reflect a metabolite-α 2μ-globulin complex.

It has been hypothesized that the nephropathy induced in male rats by TMP is the result of a stable Schiff base product formed in the liver between an aldehyde metabolite of TMP and the ε-amino acid of lysine in the α 2μ-globulin protein (Gibson and Bus, 1987) Accumulation of this altered protein in the kidney would then result due to resistance of this altered protein to lysosomal degradation. The formation of a Schiff base with an aldehyde metabolite is unlikely, however, since it has been shown recently that no marked covalent interaction between TMP or TMP metabolite and renal proteins could be demonstrated (Lock et al., 1987; Loury et al , 1987) However, evidence of

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reversible binding between the 2,2,4-trimethyl-2-pentanol and a renal protein fraction containing a 2μ-globulin has been reported (Lock et al , 1987)

The studies described above which investigated the toxicity and metabolism of TMP have been done *in vivo* and have utilized the less toxic isomer of TMP (2,2,4-TMP), few studies have used the more potent 2,3,4-TMP isomer. A recent study by Loury et al (1987) utilized *in vitro* methods to investigate viability, a 2μ-globulin production, and radiolabeled 2,2,4-TMP distribution. However, the cytotoxicity of solubilized TMP and the production of TMP metabolites by the liver *in vitro* have not been investigated.

The objective of this study was to isolate and establish purified primary cultures of male rat hepatocytes suitable for experimental exposure to sublethal concentrations of solubilized 2,3,4-TMP Experiments were conducted to evaluate the cytotoxicity and metabolism of solubilized TMP in media containing or lacking protein, to determine whether or not the presence of protein in the culture medium affects 2,3,4-TMP cytotoxicity and metabolite production These studies were designed to assist in the characterization of the nephropathy that results from exposure to hydrocarbons and to aid in the assessment of the risk to personnel working with these compounds.

MATERIALS AND METHODS

Chemicals

Ninety-nine percent pure 2,3,4-TMP was purchased from Wiley Organics (Columbus, OH). Lactate dehydrogenase test kits were purchased from DuPont Diagnostics (Hoffman Estates, IL). All media and reagents, unless otherwise stated, were purchased from Sigma Chemical (St Louis, MO).

Liver Perfusion and Hepatocyte Isolation

Livers were perfused with two different media for blood removal and digestion, respectively. Blood removal was accomplished by perfusing the liver with a modified Hank's balanced salt solution (HBSS, calcium and magnesium free, without phenol red, Gibco, Grand Island, NY) supplemented with bovine serum albumin (BSA, 500 g/mL, Fraction V), heparin (2 0 U/mL), and sodium bicarbonate (2.2 mg/mL) This perfusion was followed, without interruption of flow, by a second modified HBSS (without phenol red, Gibco, Grand Island, NY) supplemented with BSA (500 g/mL), sodium bicarbonate (2 2 mg/mL), and collagenase (Type IV; 0 3 mg/mL ~550 U/mg)

Primary hepatocytes were isolated from untreated, adult male Fischer 344 rats using the procedures of Berry and Friend (1969), as described by Bonney (1974) and Oldam et al (1979), with the following modifications Livers were perfused, at a flow rate of 35 to 40 mL/min, using a 22-gauge in-dwelling catheter (Central Inc , Westminster, MA) secured with sutures and a bulldog clamp. After perfusing the liver with the two types of perfusion media in tandem, the liver was

removed to a sterile 100-mm glass petri dish containing ~25.0 mL of the digestion medium used in the perfusion procedure and single cells were released by gentle combing with a 50-mm dog comb

Hepatocyte Enrichment and Culture

Leibovitz L-15 tissue culture medium without albumin (L-15A) and a modified HBSS medium for washing primary hepatocytes (HBSSA) were prepared according to the method of Kreamer et al. (1986) L-15A was modified by the addition of Sigma Media Supplement (10 mL/L results in a final concentration of 5.0 µg insulin/mL, 5.0 µg transferrin/mL, and 5.0 ng sodium selenite/mL), which was added aseptically to sterile L-15A the day the medium was to be used (pH 7.2 to 7.4). HBSSA was modified by the addition of BSA (0.5 gm/L; pH 7.2 to 7.4). Primary hepatocytes were enriched using low-speed, iso-density Percoll (Pharmacia, Piscataway, NJ) centrifugation as described by Kreamer et al (1986). The typical yield of primary hepatocytes (after Percoll centrifugation) from 250 to 300 g rats was 2 to 4 x 10^8 cells, with viabilities greater than 90%. Isolated hepatocytes were washed twice with HBSSA and then were resuspended to a desired concentration (approximately 5 x 10^5 cells/mL) in L-15A culture medium lacking or containing 0.5% albumin. Four milliliters of the resultant cell suspension were then added to appropriately labeled sterile 25-mL Erlenmyer flasks (Wheaton, Millville, NJ). Because of the volatility of TMP, incubations were conducted in tightly capped, Teflon®-lined flasks on a clinical rotator at 90 rpm at 37°C (95% oxygen/ 5% carbon dioxide) for 4 h.

Determination of EC_{50}

The effective concentration that resulted in 50% leakage (EC_{50}) of the total cellular lactate dehydrogenase from primary hepatocytes exposed to TMP was determined by exposing hepatocytes to selected concentrations of TMP ranging from 7.9 mM to 31 5 mM. TMP was solubilized 1:1 by volume in acetone. Subsequent TMP dilutions were made in acetone so that the final concentration of acetone in the medium remained constant at 0.5% while the TMP concentration varied. Both a negative control and a 0.5% acetone (vehicle) control were included. Replicate flasks of exposed and control hepatocytes were incubated for 4 h. The percent viability was determined for all control and treated cells by trypan blue-exclusion hemacytometer cell counts, and lactate dehydrogenase (LDH) leakage was assayed as described below. The remaining cell suspensions in the replicate flask from each treatment group were pooled and pelleted by centrifugation at 50 x g for 5 min. Pelleted cells were resuspended in a glutaraldehyde fixative and stored at 4°C for transmission electron microscopic (TEM) examination The EC_{50} was obtained by statistical analysis (see below) of the LDH leakage data from control and TMP-exposed hepatocytes. Based on the EC_{50}, a concentration of TMP below the EC_{50} was chosen for TMP metabolism studies.

Lactate Dehydrogenase Assay

One-half milliliter samples from each flask, in both control and treatment groups, were removed at 0- and 4-h time periods and placed into appropriately labeled 2-mL capped centrifuge tubes and centrifuged at 7000 x g for 5 min. After centrifugation, 0.4 mL of the supernatant from each 2-mL tube was removed and added to a second set of appropriately labeled 2-mL capped centrifuge tubes. These samples were stored at 4°C and assayed within 24 h. Samples were diluted 1:4 (v/v) with distilled water and then assayed by a DuPont ACA V discrete clinical analyzer for LDH activity (DuPont, Hoffman Estates, IL). The LDH data were expressed as a percentage of the total intracellular content. This was determined by lysing control cells (~5 x 10^5/mL in 4 mL) using 0.6% Triton-X 100, and measuring the LDH level in replicate flasks. The data were corrected for normal (positive and vehicle control) cell leakage that occurred during concurrent incubations. This was accomplished by subtracting the average of the control LDH leakage over 4 h both from the LDH content in the medium of replicate treated flasks and from the total LDH content in the flasks lysed with Triton-X 100. Only experiments in which LDH leakages from the positive and negative control groups that were less than 25% of the total intracellular LDH were subjected to statistical analysis. The corrected data were expressed as a percentage of the average adjusted total intracellular LDH content.

Cytochrome P_{450} Assay

Cytochrome P_{450} was determined with a modification of the procedure of Omura and Sato (1964) using an extinction coefficient of 91 mM^{-1} cm^{-1} for the carbon monoxide difference spectrum between 450 and 490 nm. Hepatocytes from suspension cultures were resuspended in phosphate buffered saline (pH 7.2 to 7.4) to a cell density of approximately 2.5 x 10^6 cells/mL in 2 mL, and sonicated on ice at maximum output for 10 s. One milliliter of sonicate was assayed per cuvette for P_{450} levels.

Metabolite Determination

Replicate flasks of hepatocytes, at a cell density of approximately 4 x 10^6 cells/mL, were exposed to a concentration of TMP which was below the experimentally derived EC_{50} value and which exhibited a moderate cytotoxic response (i.e., a significant increase in LDH leakage over the control groups) over 4 h. Cell suspensions then were pooled and centrifuged at 50 x g for 5 min, and the supernatants and cells stored at -20°C for later extraction of metabolites. Samples were extracted as previously described (Henningsen et al., 1988, Olson et al., 1985). Methylene chloride extracts of the hydrolyzed hepatocyte cell culture supernatants were analyzed on a gas chromatograph (GC) equipped with a flame ionization detector (model 6500, Varian, Sunnyvale, CA). Further metabolite identification was performed using a Hewlett-Packard 5985 gas chromatograph/mass spectrometer

(GC/MS; Avondale, PA) system, as previously described (Yu et al., 1987). Cellular and supernatant metabolites were identified by a comparison of GC retention times and mass spectrometry fragmentation patterns with those of authentic standards.

Electron Microscopy

Pelleted cells were fixed for TEM by resuspension in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1M cacodylate buffer at pH 7 4. After a minimum fixation of at least 24 h, the cells were post-fixed with 2% osmium tetroxide in 0.1M cacodylate buffer at pH 7 4. Cells were processed into Poiybed 812 plastic capsules. One-micron thick sections were cut in order to verify that cells were intact and suitable for thin sectioning. Thin sections (60 to 90 nm) were cut on an ultramicrotome (Ultracut E, Reichert-Jung, Cambridge Instruments Inc., Buffalo, NY) using a diamond knife. Thin sections, stained with uranyl acetate and lead citrate, were examined with a TEM (100B, JEOL USA, Inc., Peabody, MA) at 60 kV Photographs of representative hepatocytes were taken at three levels of magnification.

Statistical Analysis

Probit analysis, using the SAS procedure "Probit" (SAS Institute Inc., 1985), was used to calculate the EC_{50} (Finney, 1971). EC_{50} values, obtained from TMP treatments under different culture conditions, were compared by probability analysis using logistic regression with ch..-square distribution

RESULTS

2,3,4-TMP Hepatotoxicity

Acetone was a suitable solvent for TMP at 0 5% concentration as determined by trypan blue dye exclusion and LDH leakage The percent viability and amount of LDH leaked into the culture medium from primary hepatocytes after 4 h of incubation of control cells and cells cultured in medium containing 0 5% acetone did not differ. When primary hepatocytes were cultured in medium containing 1 0% acetone, an increase in LDH leakage and blebbing of the plasma membrane at the light microscopy level were observed However, no effect on viability was observed

Primary hepatocyte suspension cultures treated with solubilized 2,3,4-TMP over a dose range of 7 9-31 5 mM TMP (0 125 to 0 5% TMP [v/v]) showed a linear, concentration-dependent reduction in cell viability and LDH leakage under two different culture conditions, one containing 0 5% albumin, the other lacking albumin However, this reduction in viability began earlier with cells exposed to TMP in medium lacking albumin (Table 5 1-1) Lactate dehydrogenase leakage from primary hepatocytes treated with TMP was found to correlate well with viability The cytochrome P_{450} (P_{450}) content of isolated primary hepatocytes from positive and negative (0 5% acetone) controls, and two

TMP treatments (7 9 and 15 7 mM), indicated that no reduction in the P_{450} content occurred over the 4-h incubation period (data not reported)

TABLE 5.1-1. VIABILITY AND LACTATE DEHYDROGENASE LEAKAGE OF PRIMARY RAT HEPATOCYTES TREATED WITH INCREASING TMP CONCENTRATIONS IN FOUR H SUSPENSION CULTURES

TMP Dose (mM)	Medium Lacking Albumin[a]		Medium Containing Albumin (0.5%)[b]	
	% Viability[c]	% LDH in cells	% Viability[c]	% LDH in cells
7.9	99	93	100	98
12.0	86[d]	85[d]	99[e]	95[e]
15.7	52	59	89	87
19.1	14[f]	28[f]	58[e]	51[e]
31.5	0	0	0	0

[a] Values represent the average mean from three experiments incorporating replicate flasks, and are expressed as percent of the total intracellular LDH content of control cells

[b] Values represent the average mean from four experiments incorporating replicate flasks, and are expressed as percent of the total intracellular LDH content of control cells

[c] Viability was determined by trypan blue dye (0 08%) exclusion using a hemacytometer, three hemacytometer counts were averaged for each flask

[d] Values reflect the average of replicate flasks from two experiments

[e] Values reflect the average of replicate flasks from three experiments

[f] Values reflect the average of replicate flasks from a single experiment

SAS Probit analysis of the LDH leakage data from experiments involving 2,3,4-TMP treatment in medium containing albumin and in medium lacking albumin indicated a difference in the predicted EC_{50} (Figure 5 1-1) The predicted EC_{50} for 2,3,4-TMP exposures in medium lacking albumin and in medium containing 0.5% albumin were 17 1 mM (0 27% (v/v)) and 20.7 mM (0 33% (v/v)) TMP, respectively When the hepatocyte LDH leakage data from 2,3,4-TMP exposures under these experimental culture conditions were analyzed by a logistic regression using chi-square distribution, a significant difference in the predicted EC_{50} values was found ($P<0.01$, the exact probability that the two values were the same)

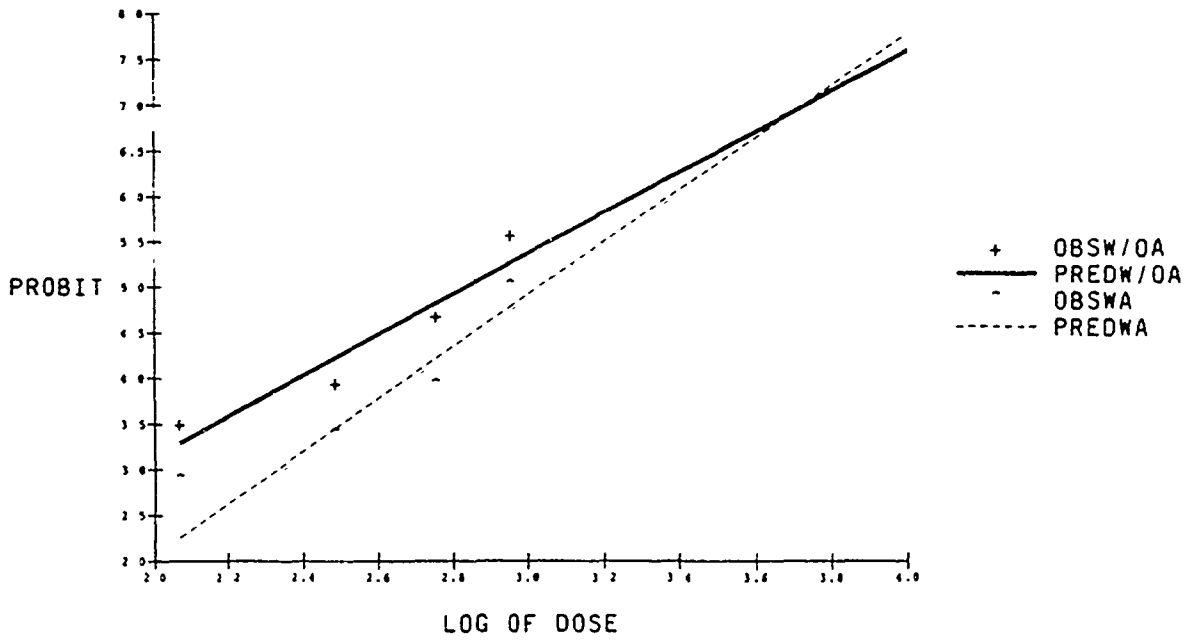

Figure 5.1-1. Predicted EC_{50} for 2,3,4-TMP Derived by SAS Probit Analysis of LDH Leakage from Primary Hepatocytes Treated with a Range of TMP Doses (7.9-31.5 mM) for 4 h in Medium Containing (0.5%) or Lacking Albumin. Observed Probit for a particular TMP dose in medium lacking albumin that was derived from three experiments (OBSWA/OA); Predicted Probit response for TMP treatment in medium lacking albumin (PREDW/OA); Observed Probit for a particular TMP dose in medium containing 0.5% albumin derived from four experiments (OBSWA); Predicted Probit response for TMP treatments in medium containing 0.5% albumin (PREDWA). The predicted EC_{50} (at Probit 5.0) from TMP treatments in medium lacking and containing 0.5% albumin were 17.1 and 20.7mM, respectively.

Electron Microscopy

At the ultrastructural level control cells were relatively normal in appearance. There was no observable difference in morphology between hepatocytes cultured in medium lacking albumin and cells cultured in medium containing albumin (Figures 5.1-2A and 5.1-2B). Vehicle (acetone) control hepatocytes in medium lacking or containing albumin showed an increase in smooth endoplasmic reticulum (SER) and a slight decrease in microvilli on the cell surface (Figure 5.1-2B). Vehicle control hepatocytes in either medium lacking or containing albumin had blebs on the cell surface but the blebs were larger in cells cultured in medium lacking albumin. The ultrastructural observations correlated with the LDH leakage data.

Hepatocytes treated with 7.9 mM TMP in medium lacking albumin (Figure 5.1-2C) had more condensed chromatin associated with a section of the nuclear membrane than is normal in nuclei. Other observable changes in morphology included: mildly swollen mitochondria, increased amounts of cytoplasmic lipid, normal to moderately dilated rough endoplasmic reticulum (RER) with some loss of ribosomes, almost complete loss of microvilli from the cell surface, occasional blebs filled with SER projecting from the surface of cells, and increased numbers of peroxisomes. Most of the TMP-exposed hepatocytes had vacuoles that were associated with degenerative changes in organelles.

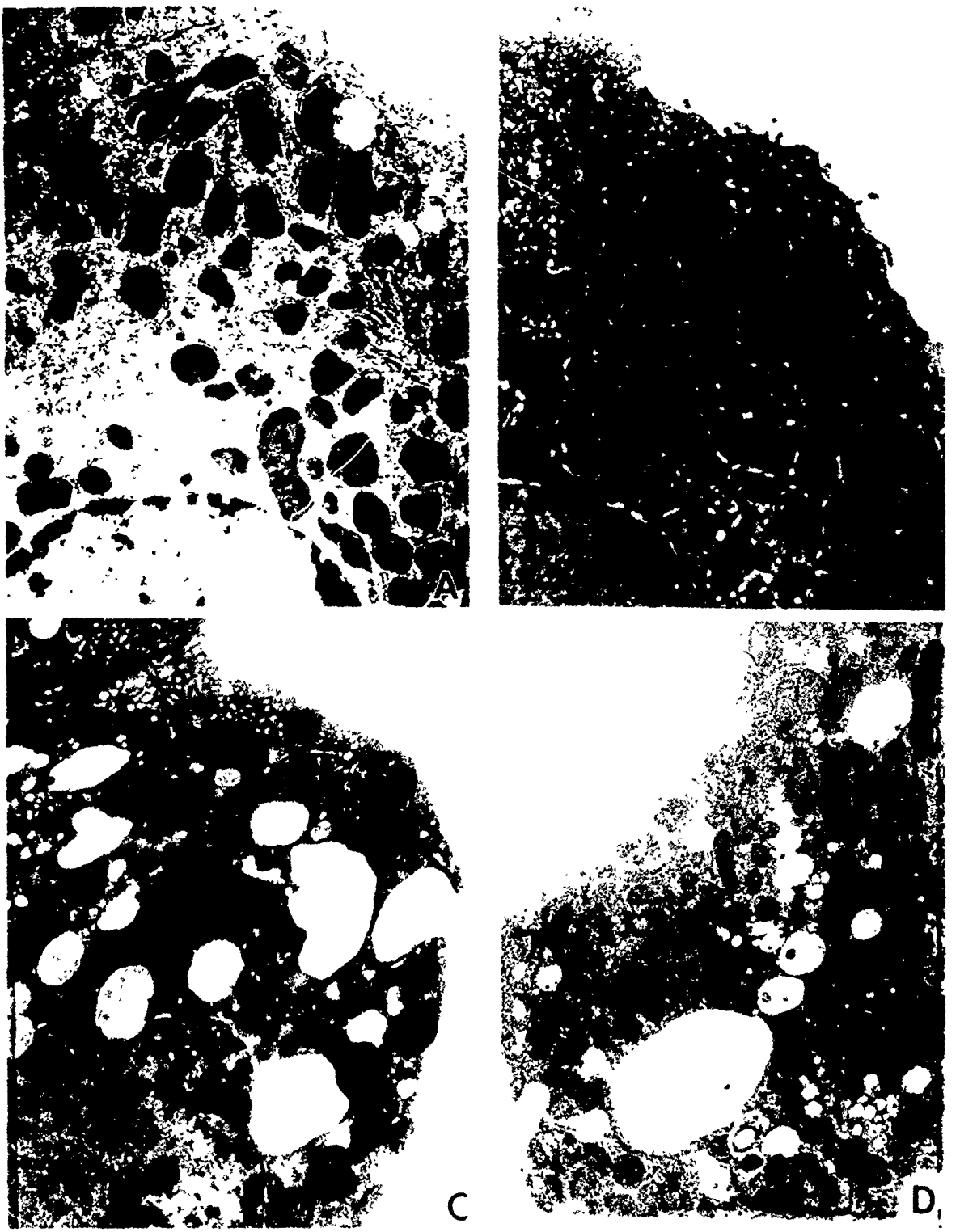

Figure 5.1-2. (A) Control Primary Hepatocyte after 4 h of Culture in the Medium Lacking Albumin. (Arrow indicates SER.) (B) Primary Hepatocyte after 4 h of Culture in the Medium Containing 0.5% Acetone and 0.5% Albumin. (Arrow indicates increased SER.) (C) Primary Hepatocyte after Treatment with 7.9 mM TMP for 4 h in Medium Lacking Albumin. (D) Primary Hepatocyte after Treatment with 7.9 mM TMP for 4 h in Medium Containing 0.5% Albumin. (Magnification 10,000 x)

Hepatocytes treated with 7.9 mM TMP in medium containing albumin (Figure 5.1-2D) did not show an increase in peroxisomes The hepatocyte cell surface changes were very similar, but not as severe, as cells treated with TMP in medium lacking albumin The degenerative changes in cell organelles were essentially the same Primary hepatocytes treated with 12 mM and 15 7 mM TMP in medium lacking or containing albumin were progressively more affected than hepatocytes treated with 7.9 mM TMP The nuclei and RER were affected to a greater degree than other organelles at the higher TMP concentrations It was also observed at higher TMP concentrations that chromatin condensation and its non-specific association with a section of the nuclear membrane continued to increase while RER became more dilated, lost ribosomes, and appeared to be breaking down in some cells. The major difference between cells cultured in medium lacking and containing albumin was the increase in peroxisomes in hepatocytes treated with TMP in medium lacking albumin.

At 19 1 mM TMP, in medium lacking albumin, there were not enough viable hepatocytes to process for electron microscopy. In contrast to this result, it was found that viable hepatocytes could be recovered for electron microscopy analysis from cell cultures treated with 19.1 mM TMP in medium containing albumin. Hepatocytes which survived treated with 19.1 mM TMP in medium containing albumin had swollen mitochondria, dilated RER, and appeared to be on the verge of necrosis.

2,3,4-TMP Metabolism

Analysis of primary hepatocyte and supernatant extracts from TMP-treated cultures indicated the presence of two metabolites by comparison with the retention times of authentic standards of TMP metabolites. A third metabolite (a carboxylic acid) was identified upon subsequent GC/MS analysis. The GC tracing and mass spectrum of the extracted supernatant from a primary hepatocyte suspension culture treated with 12.0 mM 2,3,4-TMP are presented in Figures 5.1-3 and 5.1-4 The three TMP metabolites identified were 2,3,4-trimethyl-1-pentanol (TM-1-P OH), 2,3,4-trimethyl-2-pentanol (TM-2-P OH) and 2,3,4-trimethyl-1-pentanoic acid (TMPA) The base peaks of these three metabolites were m/z 70 (M-60), m/z 59 $[M-C_4H_7O]^+$, and m/z 74 (M-70), respectively

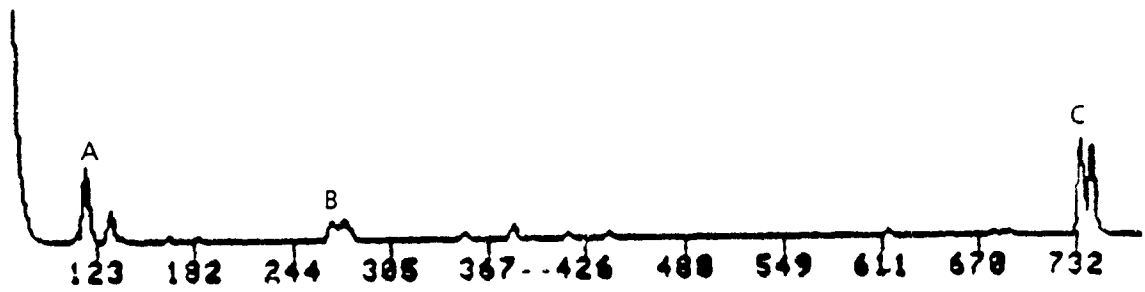

Figure 5.1-3. Representative GC Tracing of Supernatant Extract from Primary Hepatocyte Suspension Cultures Treated with 2,3,4-TMP for 4 h at 37°C. (A: 2,3,4-trimethyl-2-pentanol; B: 2,3,4-trimethyl-1-pentanol; C: 2,3,4-trimethyl-1-pentanoic acid)

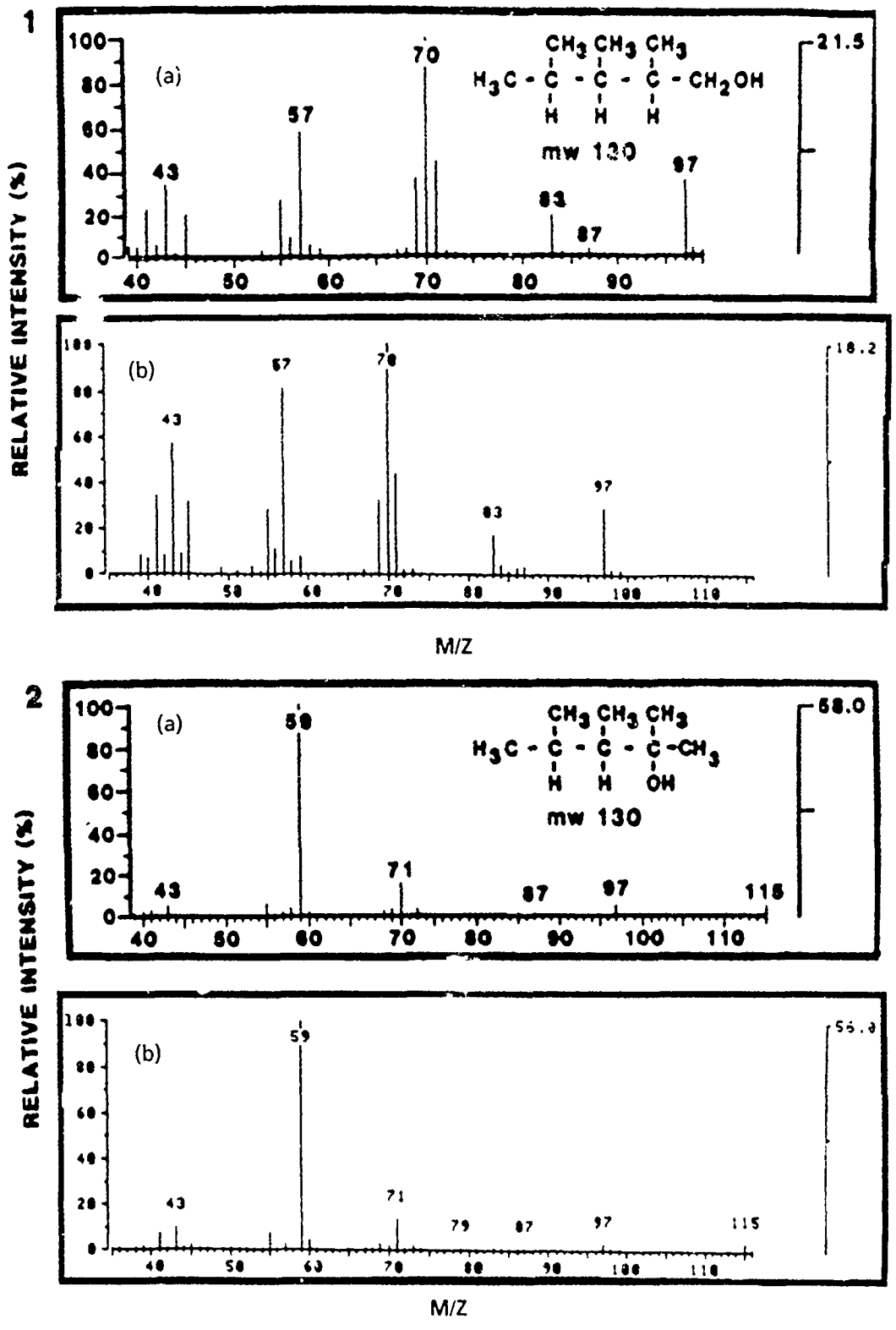

Figure 5.1-4. Mass Spectra of 1 (a) 2,3,4-Trimethyl-1-Pentanol*, (b) medium extract; 2 (a) 2,3,4-Trimethyl-2-Pentanol*, (b) medium extract. (*Taken from Yu et al., 1987)

(continued)

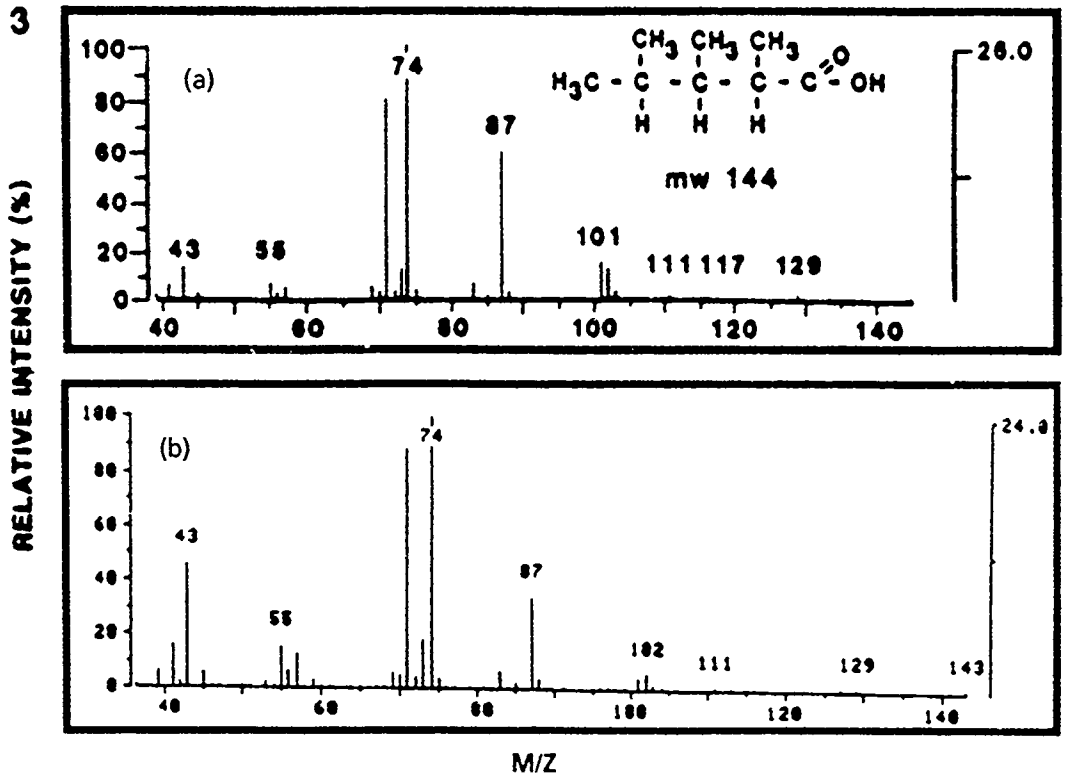

Figure 5.1-4. (Continued). 3 (a) 2,3,4-Trimethyl-1-Pentanoic Acid*, (b) medium extract. (*Taken from Yu et al., 1987)

DISCUSSION

2,3,4-TMP was toxic to primary hepatocytes, causing progressively more severe degenerative changes with increasing concentration of TMP in medium both lacking and containing albumin. These degenerative changes were found to be more severe in medium lacking albumin than in medium containing albumin at identical TMP doses. Albumin in the culture medium appeared to protect the cells and prevented the increase in peroxisomes seen in hepatocytes cultured in medium lacking albumin. The significance of this result is not known at this time.

Evidence that albumin protected primary hepatocytes from the effects of TMP was shown by a decreased number of cell membrane changes in hepatocytes cultured with albumin and the presence of a sufficient number of viable cells at 19.1 mM TMP on which to perform electron microscopy. The first point is in agreement with the LDH leakage data while the second point is in agreement with the viability data These data indicate that either the albumin helps to stabilize the plasma membrane or binds to TMP resulting in treatment of hepatocytes to a lower concentration of TMP.

It has been suggested that TMP or its metabolites bind to a male-specific protein and that this interaction accounts for the increased protein droplet nephropathy observed in the renal cortex of

male rats exposed to TMP (Charbonneau et al., 1987; Lock et al , 1987, Loury et al., 1987). In this study, it was found that there was a significant increase in the predicted EC_{50} when primary hepatocytes were treated with 2,3,4-TMP in medium containing 0 5% albumin compared to exposures in medium lacking albumin. This increase is in agreement with the TEM data which indicates these results may be due to binding of TMP or its metabolites to albumin protein or due to an albumin-mediated membrane stabilization The latter seems more likely, since control primary hepatocytes in medium containing 0.5% albumin had reduced LDH leakage over four h of incubation than cells incubated in medium lacking albumin

GC/MS analysis of an extract of medium lacking albumin from a 2,3,4-TMP treatment identified three metabolites (2,3,4-TM-1-P OH, 2,3,4-TM-2-P OH, and TMPA) These three metabolite were identical to those previously identified in the urine of male rats exposed to 2,3,4-TMP (Yu et al , 1987) TM-2-P OH and TMPA appear to be the predominant metabolites formed This corroborates the findings of Charbonneau et al (1987), who found that the 2,2,4-TM-2-P OH metabolite was the major metabolite present in the male rat kidney This result strongly suggests that the TM-2-P OH metabolite is responsible, in part, for the observed TMP-induced nephropathy The apparently lower amounts of the TM-1-P OH metabolite probably reflects its further oxidation to the TMPA metabolite. The toxicity of the TMPA metabolite has not been investigated

In a recent metabolic distribution study by Charbonneau et al (1987) involving ^{14}C-labeled 2,2,4-TMP, the highest tissue concentrations of TMP or its metabolites (expressed as a percentage of dose) occurred in the kidney and liver 12 h after a single dose This report provides the first direct evidence of the involvement of the liver in the metabolism of 2,3,4-TMP Metabolism of 2,3,4-TMP by the kidney cannot be ruled out, however, since it also possesses a P_{450} system Analysis of metabolite production using primary kidney proximal tubules to compare with those produced by the liver would establish the role of the kidney in the metabolism of TMP (study in progress).

Recently, Loury et al (1987) treated primary hepatocyte cultures with 0 5% (v/v) of the non-water-soluble 2,2,4-TMP by direct incorporation of the neat 2,2,4-TMP into the medium and found that 77% of the attached cells were viable after 15 h The results presented in this report indicated that soluble 2,3,4-TMP at 0 5%, in medium lacking or containing 0 5% albumin, resulted in total lysis of primary hepatocytes suspension cultures after four h of incubation Although these results seem to corroborate the *in vivo* findings of Hobson et al (1985), that the 2,3,4-TMP isomer is more toxic than the 2,2,4-TMP isomer, solubilization of TMP may result in more direct exposure of the cells to the TMP in the medium than would occur by incorporation of neat TMP It is also possible that the increased toxicity of the solubilized 2,3,4-TMP may have been augmented by the presence of the acetone solvent. Furthermore, suspension cultures may be more sensitive to toxic insults than attached cells

The results of this study indicate that albumin confers a protective effect against TMP cytotoxicity This protection is more likely the result of stabilization of the hepatocyte plasma membrane rather than binding of TMP or its metabolites to albumin. Experiments have been initiated to test the cytotoxicity and metabolism of 2,3,4-TMP using primary male rat kidney proximal tubules for comparison with the results from the liver work.

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5.2 METHODS FOR GENERATING AND STUDYING AEROSOL DISSOLUTION PROPERTIES: BeO CONTAINING ROCKET EXHAUST PARTICLES

R.L. Carpenter, K.L. Yerkes, E.C. Kimmel, A. Vinegar, C.D. Flemming, and W.R. Sayers

INTRODUCTION

In order to aid in the translation of laboratory experiments into real world human experience, mathematical models describing the relationships between toxicant dose and biological response are under development These models have been limited to vapors and gases However, description of the inhaled dose of materials that are solids, such as beryllium oxide (BeO), required the ability to model deposition and clearance of particulate material from the lungs. Such models have been under development. In this study, the description developed by Wojciak (1988) has been reformulated and extended to include mechanisms of clearance and particle dissolution. The methods developed are generally applicable to liquid and solid particles. The techniques developed to date have used beryllium-bearing materials as test substances but will be continued using other materials whose properties meet analytical, modeling, or toxicological needs

Although much is known about the toxicity of beryllium (Be) and its oxide, it is currently unclear how this information is related to the material produced from the combustion conditions inside a rocket motor that uses Be as a fuel. In general, ingestion and inhalation are considered to be the major routes of exposure for humans and animals. Oral exposure to environmentally relevant levels of Be compounds has not been shown to produce significant toxicity (Callahan, 1979). However, both acute and chronic toxic effects are recognized in humans and animals following inhalation of various forms of Be, including BeO, from occupational exposure or from laboratory experiments. Acute inhalation of aerosols having a high soluble Be content leads to inflammation of the respiratory tract and pulmonary edema. A few cases of acute pneumonitis following repeated human exposure to low levels of Be have been reported (Wilbur, 1980). Berylliosis was first described in fluorescent lamp workers in the 1940s (Hardy and Tabershaw, 1946). This disease is a chronic immunologically mediated disease that results in a granulomatous hypersensitivity response in the lung. Be carcinogenicity is well-established in several species of laboratory animals, but the human cancer risk is not well-established (Wagoner et al., 1980)

The severity of BeO toxicity apparently is a function of the temperature at which the BeO is formed. BeO prepared at low temperatures (approximated 500°C) appears to be more toxic and carcinogenic than material produced at temperatures of 1600°C (Spencer et al , 1968). It has been suggested that differences in solubility may account for this difference in toxicity. Therefore, measures of the relative solubility of both high- and low-fired BeO as well as rocket exhaust materials could provide a basis for comparing the biological availability of Be from rocket exhaust material to that of BeO A method for measuring the relative solubility of fine particles has been developed

(Miglio et al , 1977) and applied to the measurement of a wide variety of insoluble aerosol particles (Eidson and Mewhinney, 1983)

MATERIALS

Three different formulations of BeO were used in this study Two of these materials were pure BeO produced by precipitation of $Be(OH)_2$ followed by calcining either at 550°C or 1600°C These materials were obtained from Brush-Wellman, Inc The third material was collected from test firings of experimental rocket motors in enclosed chambers All three materials were passed through an air-driven Trost jet mill to reduce particle diameter prior to use

METHODS

Particle Dissolution

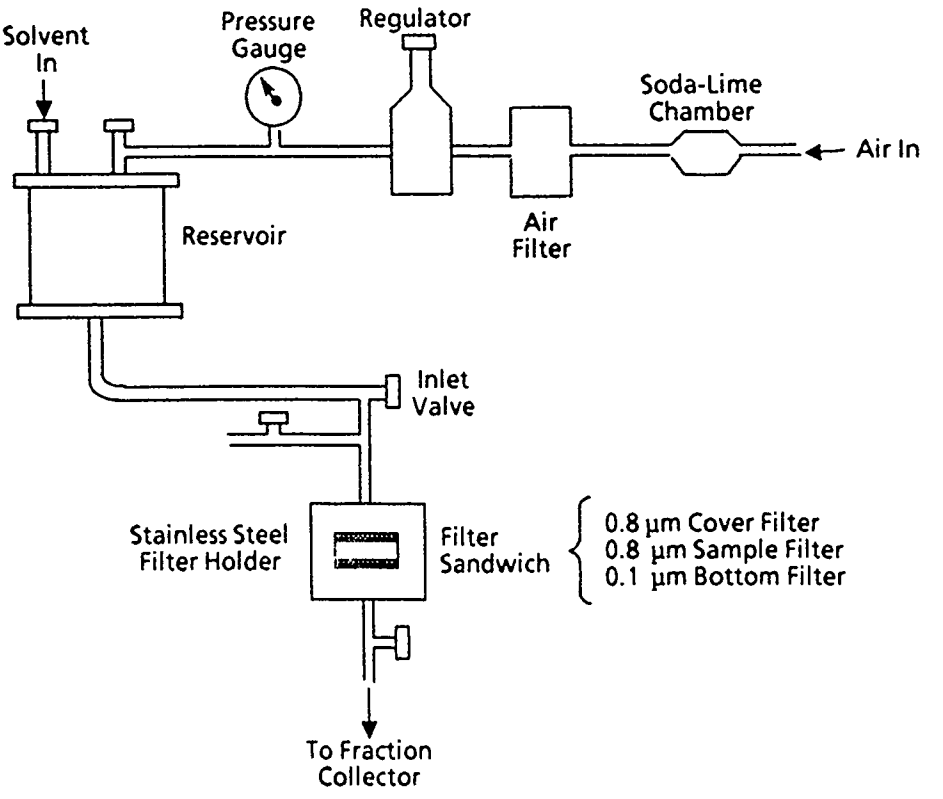
Particle solubility was determined by the method of Kanipilly et al. (1973). Figure 5.2-1 illustrates the flow through filters used in this study These filters were loaded with approximately 50 mg of BeO powder and placed in a water bath at 37°C As shown in Figure 5 2-1, lung serum simulant was metered through each filter holder at a flow rate of 21 mL/h from a sterile reservoir and collected in a fraction collector. The lung serum simulant was prepared using the formula in Table 5.2-1. Samples were taken from the fraction collector daily and analyzed for Be content.

Dissolved Be initially was determined by a chleation method that used trifluoroacetylacetone and subsequent gas chromatography Experience indicated that sufficient water was carried through the preparation procedure to degrade the capillary column used for chromatography The alternate analysis method of atomic absorption spectroscopy was used to determine the fraction dissolved per day for the low-fired sample A second chromatographic method using selected ion monitoring on the Hewlett-Packard mass selective detector was developed using a short column with lower degradation This method was used for initial measurements of the remaining dissolution samples.

Lung Model for Particles

Particle deposition in the lung for both inspiration and expiration can be calculated using the formulation of Wojciak (1988) However, such a model provides no mechanisms for particle removal from the lung. The best understood mechanism for clearing particles from the lung is removal of deposited material by movement of mucus up the airways Measurements have been made of the mucous transport rates of the upper airways of laboratory animals (Felicetti et al , 1981) Similar data are available for 16 generations of the human lung (Hardy and Pasternack, 1972, Lee et al , 1979) Particles also clear from the lung by mechanisms other than mucociliary clearance These mechanisms include dissolution of the deposited particles (Task Group on Lung Dynamics, 1966) and phagocytosis by macrophages, followed by transport to the ciliated airways (Mueller and Guilmette, 1987) These concepts are being formulated in an overall model of deposition and clearance based on lung physiology Once validated against laboratory data, the model will be useful in experimental design and analysis of data

EXPERIMENTAL ARRANGEMENT



PARALLEL FLOW FILTER HOLDER

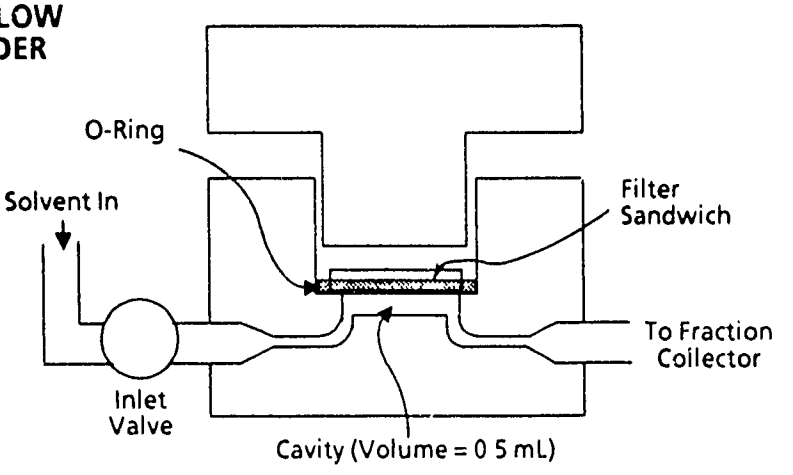


Figure 5.2-1. Schematic of Flow System and Filter-Holder Assembly.

TABLE 5.2-1. COMPOSITION OF BLOOD PLASMA AND LUNG FLUID SIMULANT

Chemical Species	Blood Plasma (mM/L)	Serum Simulant (mM/L)
Sodium	142 0	145 0
Potassium	5.0	–
Calcium	2.5	0 2
Magnesium	1 5	–
Ammonium	–	10.0
Protein	6.6	–
Chloride	103 0	126 0
Bicarbonate	27 0	27 0
Phosphate	1 2	1 2
Citrate	0.2	0 2
Organic Acid	6.0	6.0
Sulfate	0.5	0.5

Chemical Compound	NaCl	NH_4Cl	$NaHCO_3$	NaH_2PO_4	Na_3Cit	Glycine	H_2SO_4	$CaCl_2$
mM/L	116	10	27	1.2	0.2	6	0.5	0.2

Inhalation Exposure

Nose-only inhalation studies were designed to provide information on the deposition, clearance, and potential for early lung damage caused by three forms of BeO. Currently, the limited availability of rocket exhaust material restricts studies using this material to a single exposure for the purpose of determining deposition and clearance

A fluid bed generator was chosen as the method for dispersing the BeO powder Figure 5 2-2 shows a schematic of a fluid bed generator as used for the dispersion of powders The generator consists of three sections. (1) a plenum chamber, (2) a bed chamber, and (3) a freeboard Fluidizing air is injected into the plenum chamber and passes through a porous metal disk providing a uniform velocity entering the bed chamber Stainless steel bed material, with which the powdered fines are mixed, is contained in the bed chamber Fluidizing air flow will disturb the bed, resulting in its fluid-like motion At the onset of fluidization, the powdered fines (BeO) will be elutriated from the course stainless steel bed Dispersed fines are transported up the freeboard and into the exposure chamber

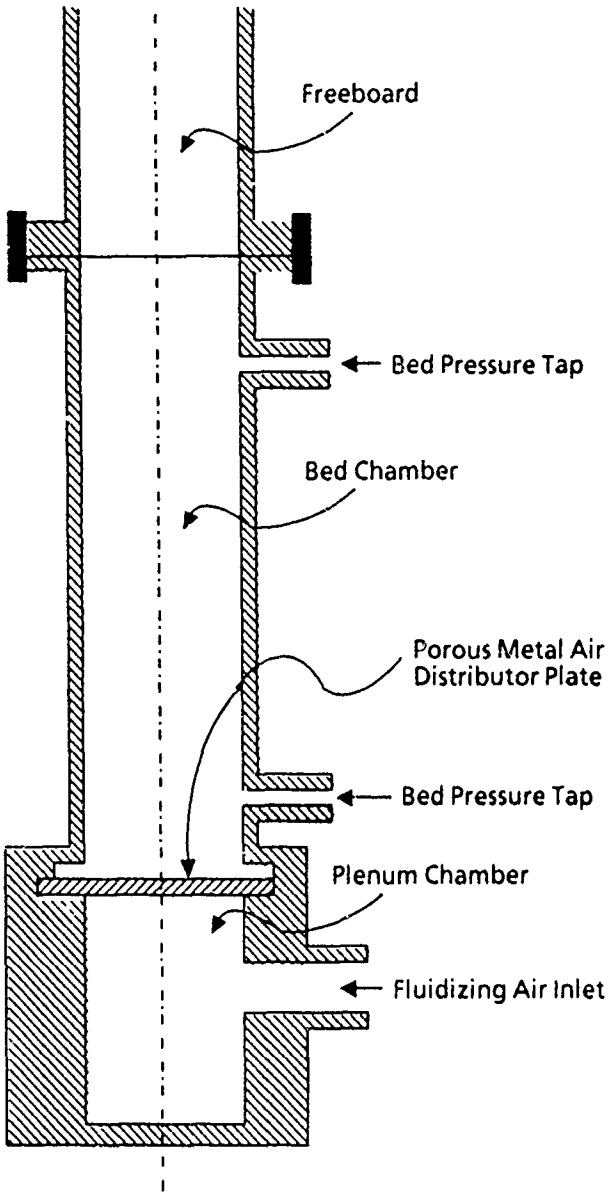


Figure 5.2-2. Cross Section of Fluid Bed Aerosol Generator.

RESULTS

Particle Dissolution

Solubility of each BeO material was determined at 37°C by collecting lung serum simulant for approximately 350 h and analyzing the collected fluid for Be. The results of these analyses are presented in Figure 5.2-3.

Lung Model for Particles

The particle deposition model of Wojciak (1988) has been recoded into Advanced Continuous Simulation Language and verified for rats. Mucous clearance rates are available experimentally in

rats only for the trachea because the small diameter of the lower airways in rodents limits experimental measurement However, this is not the case for humans The available data for human mucous clearance that is complete for all generations of ciliated airways has been used to estimate the lower airway clearance rates for rats The human clearance rates were reduced proportionately until the tracheal clearance rates agreed with that observed in the rat These reduced clearance rates were included to describe clearance at each generation of the ciliated airways

Currently, clearance calculations are limited to clearance of particles from the tracheal-bronchial regions by way of mucociliary action Initial model development also has included whole-lung particle dissolution for relatively soluble materials and only for the nonciliated airways if the particles are relatively insoluble. Data for validating these portions of the model are not available from the literature. The model does not account for the movement of macrophages from the non-ciliated airways to the ciliated airways since no information is available on the dynamics of this mechanism

Inhalation Exposure

The fluidizing characteristics of the fluid bed aerosol generator were determined for two bed heights (1 in and 2 in). By observing the pressure drop across the bed with increasing superficial velocity, the minimum fluidizing velocity (MFV) can be determined Initiation of fluidization was distinct, occurring when the bed unlocked and causing the pressure drop across the bed to drop from a maximum. Under poor fluidization conditions, these pressure-velocity curves show oscillations and poor distinction of the MFV Figures 5 2-4 and 5 2-5 show the bed pressure drop with increasing superficial velocity for the two bed heights investigated

DISCUSSION

Solubility Studies

Figure 5 2-3 indicates that the rocket exhaust particles are more soluble than either the high- or low-fired BeO materials. Insufficient data are available at this time to determine the dissolution properties of the high-fired BeO, but the dissolution curve for the low-fired material suggests that the material is polycrystalline and changes dissolution rate as the particle surface area changes Similar behavior is not evident in the case of the rocket exhaust material

Model Development

A simulation model has been written for particle deposition, mucociliary clearance, and dissolution in the lung This model can be refined when information describing macrophage clearance becomes available Data are also needed on the short-term clearance of material from the lung in order to exercise the mucociliary clearance features of the model

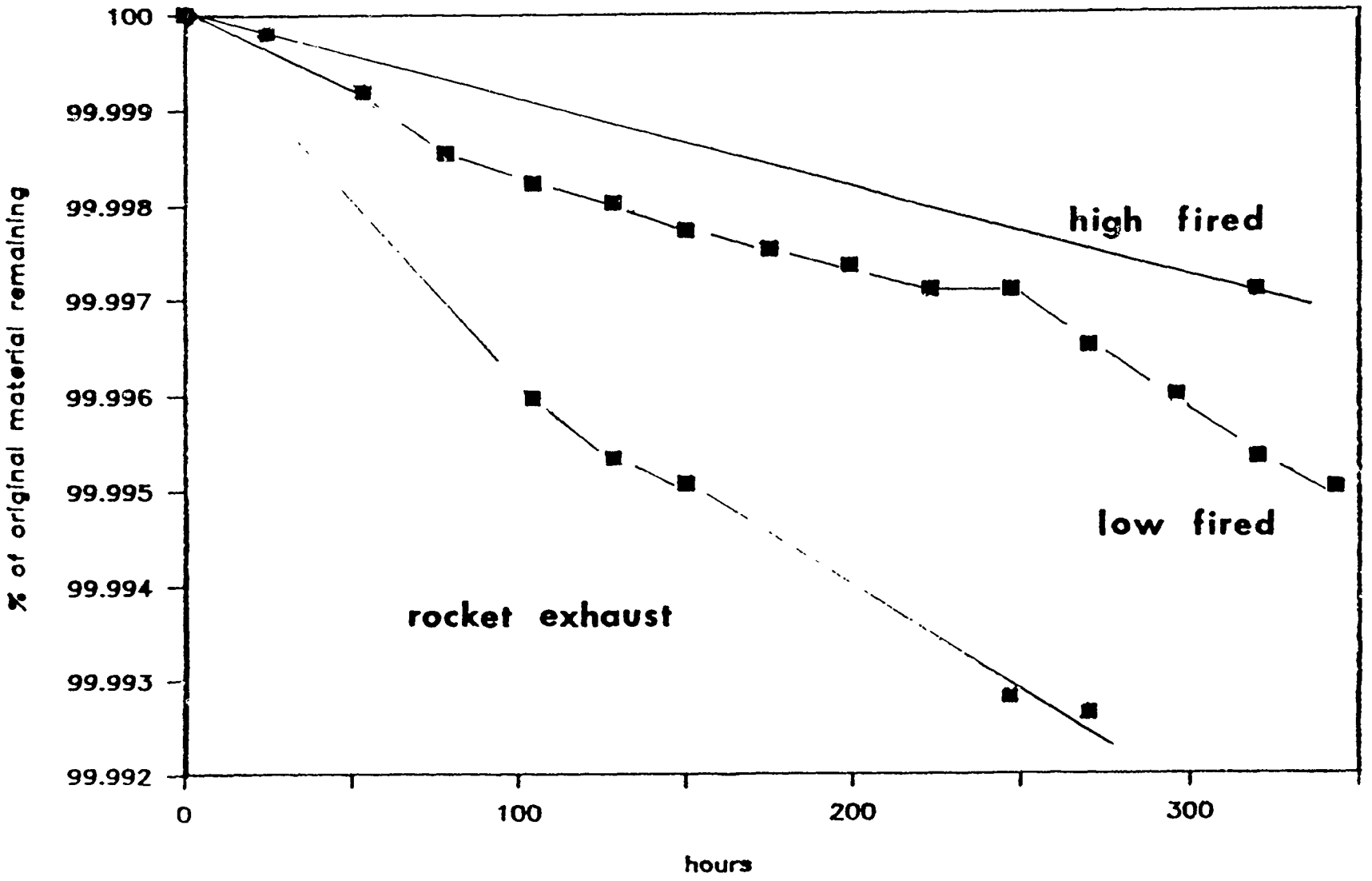


Figure 5.2-3. Beryllium Oxide Dissolution in Simulated Lung Fluid at 37°C.

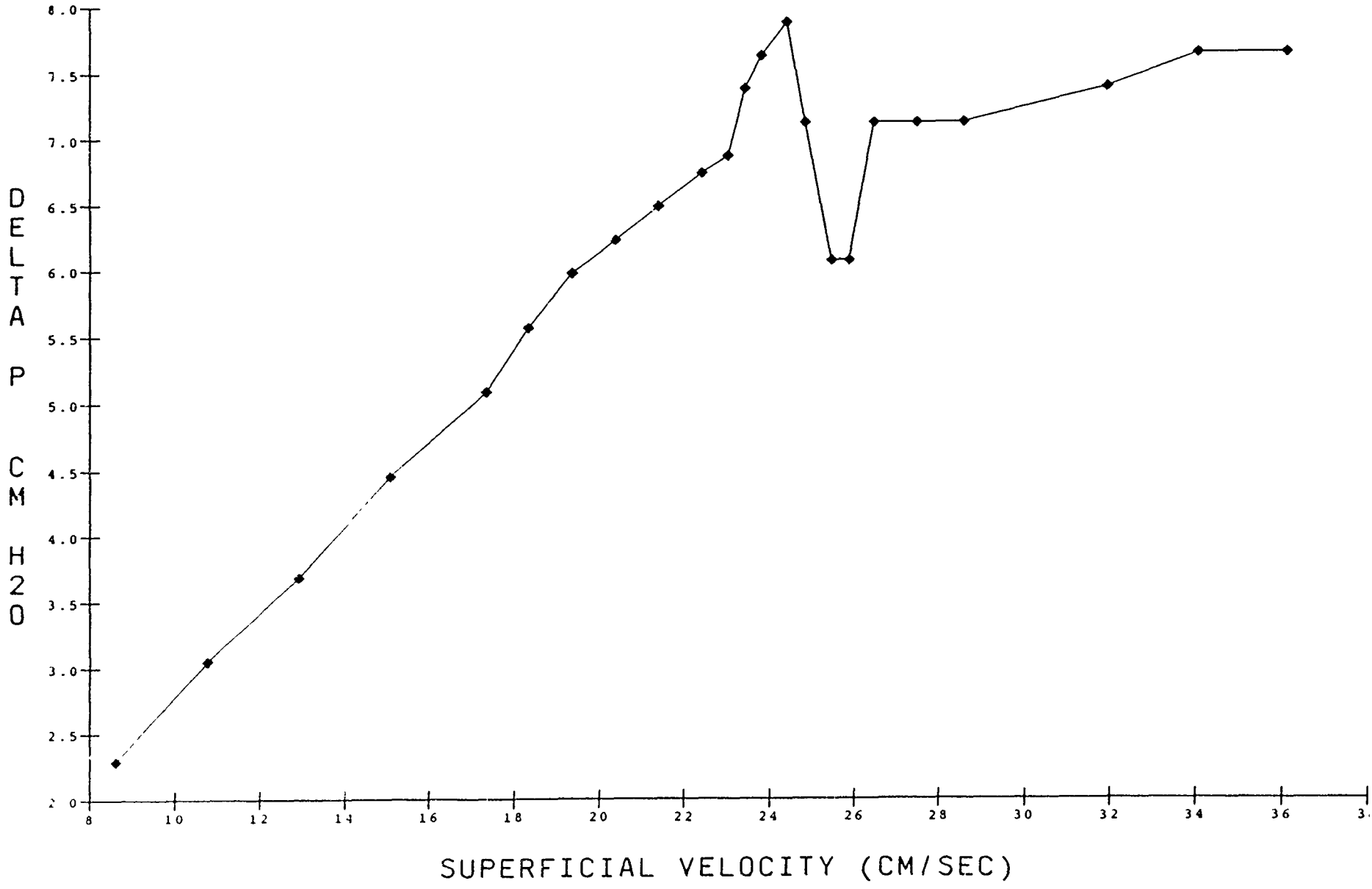


Figure 5.2-4. 1" Fluid Bed Pressure-Flow Plot (2" Bed Depth).

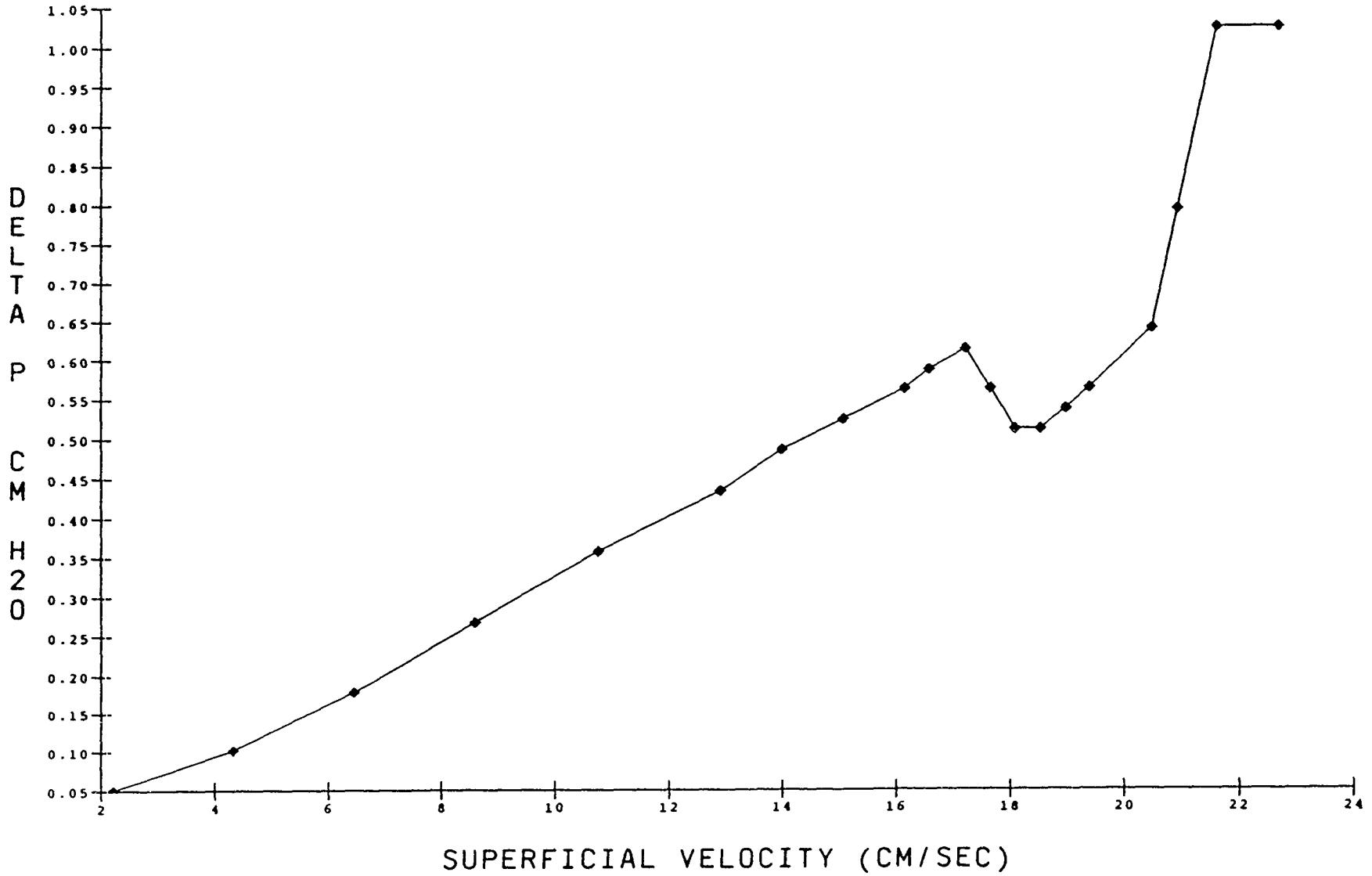


Figure 5.2-5. 1" Fluid Bed Pressure-Flow Plot (1" Bed Depth).

Inhalation Exposures

The 2-in deep bed appeared to have acceptable fluidizing characteristics with a well-defined MFV and no pressure oscillations Lowering the bed height to 1 in. gave a poorly defined MFV in addition to a sudden increase in pressure drop across the bed at the onset of fluidization

The 1-in fluid bed generator was incorporated into the exposure system as shown in Figure 5 2-6. The system consists of an 80-port nose-only exposure chamber with sampling capabilities located at the exhaust outlet and a sample port located in the chamber Particle sampling at the exhaust includes a multi-cyclone train to recover the aerosolized BeO powders in discrete particle size fractions and a cascade impactor for aerodynamic particle sizing during animal exposures Additional filter samples and electrostatic precipitator samples could be taken at the chamber sample port and used for determining mass concentration or for electron microscopic analysis of collected particles.

These methods will be used to gather data on the mechanisms that currently limit model development. The existing model will be refined as research results become available, with the goal of developing a model capable of relating inhaled dose to body tissue burdens of aerosols deposited in the lung.

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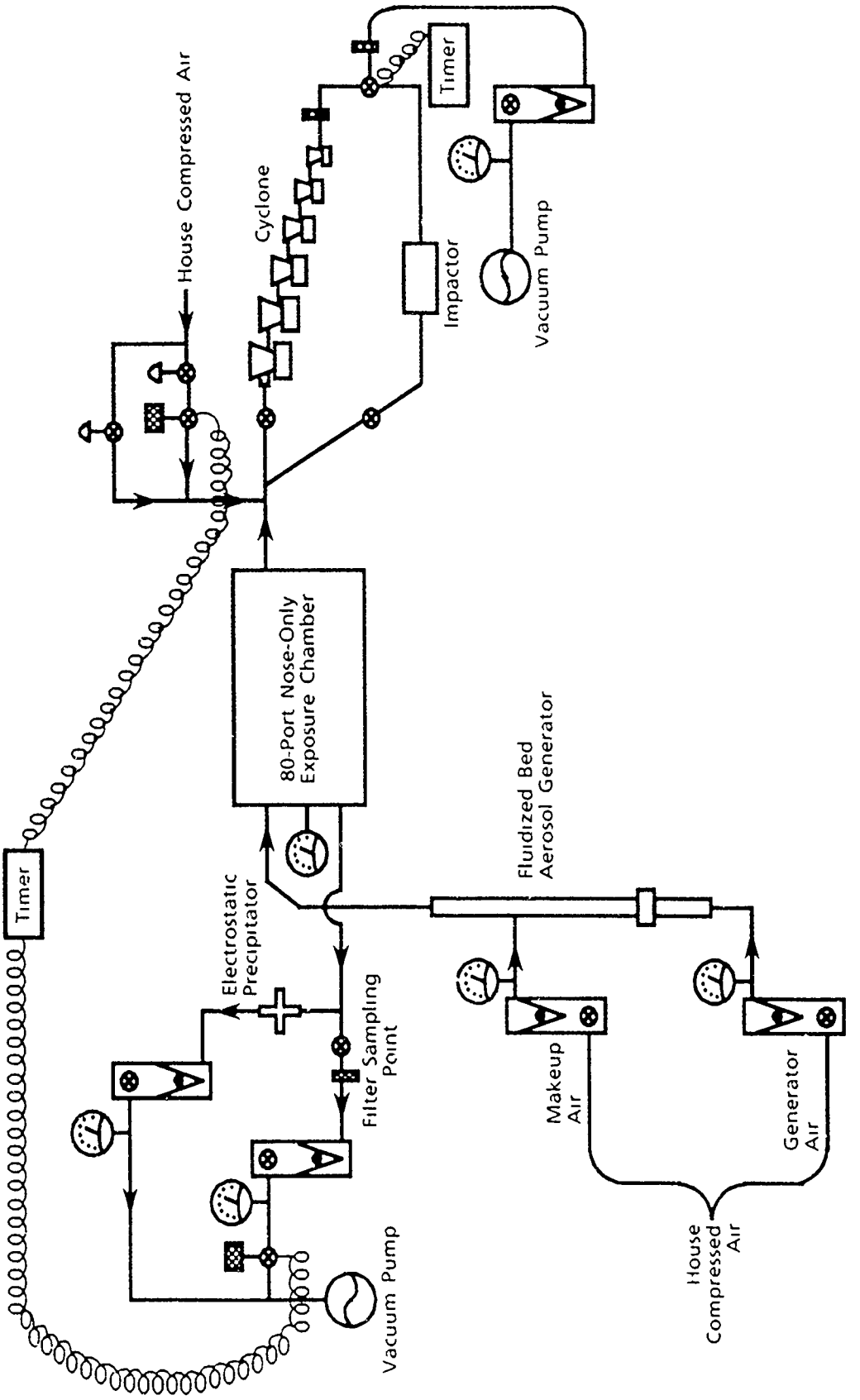

Figure 5.2-6. Schematic of Exposure System with 1-in. Fluid Bed Generator.

5.3 ASSESSMENT OF THE GENOTOXIC POTENTIAL OF BERYLLIUM OXIDE ROCKET EXHAUST USING CULTURED RESPIRATORY EPITHELIAL CELLS

V.E. Steele[a], B.P. Wilkinson[a], J.T. Arnold[a], and R.S. Kutzman

INTRODUCTION

There are technical advantages in the utilization of beryllium (Be) solid propellant as fuel for rocket motors. However, an exhaust product (BeO), is of primary toxicological concern. An apparent difference between the toxicities of BeOs produced at low and high temperature-firing has been reported, with the oxide produced at the higher temperature being less toxic (Spencer et al., 1968). It also appears that the material produced in the exhaust of Be-fueled rockets is more like that produced at high temperature.

In previous studies, BeO and Be phosphate were shown to cause lung carcinomas and bone sarcomas by inhalation, and by intravenous and intraosseous administration (International Agency for Research on Cancer, 1980). In the studies described here, the carcinogenic and genotoxic properties of rocket exhaust particles containing Be were evaluated by using rat tracheal epithelial (RTE) cells, by measuring (1) the morphological transformation of these cells as an indicator of oncogenic transformation (Steele and Mass, 1985), and (2) the DNA single-strand breaks resulting from such exposures. RTE cells also were exposed to low and high temperature-fired BeO particles within the same experiments for comparative analysis. The relative amount of DNA single-strand breaks was measured using an alkaline elution technique (Kohn et al., 1976). In these studies, RTE cells were used as a model for human respiratory tract epithelial cells (a major target tissue) to provide data for evaluating the possible carcinogenic potential of rocket exhaust particles that contain Be

MATERIALS AND METHODS

The rocket exhaust residue sample was obtained from Morton Thiokol, Inc., and is referred to as Be1 in this report. The low-fired BeO sample was produced by Brush Wellman, Inc , and is referred to as Be2 herein The high-fired BeO also was produced by Brush Wellman, Inc , and is referred to as Be3. The test samples were prepared as previously described (Steele and Kutzman, 1988)

Tracheal epithelial cells were isolated by aseptically removing the tracheas from 8- to 12-week-old male Fischer 344 rats The animals were first euthanatized by CO_2 asphyxiation and

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exsanguination. Then the tracheas were cannulated with sterile polyethylene tubing and rinsed with Joklik's modified minimum essential medium (JMEM) The tracheal lumens were then filled with a protease solution (Pronase, Type XIV, Sigma Chemical Co., Inc.) and incubated overnight in a refrigerator at 4°C. The following day, the epithelial cells were rinsed from the tracheas with cold Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were filtered to remove clumps, centrifuged, and resuspended in culture medium as described below

For the RTE Transformation Assay approximately, 20,000 cells were placed into each collagen-coated, 60-mm tissue culture dish. The cells were cultured in a 1:1 mixture of Ham's F-12 medium and 3T3-conditioned DMEM + 2% FBS, containing insulin, hydrocortisone, transferrin, and bovine hypothalamus extract (Steele et al., 1984). At Day 1, the cultures were treated for 24 h with 30, 10, 3, 1, or 0 3 µg BeO/mL of culture medium. These concentrations were selected using a range-finding assay (Steele and Kutzman, 1988). The treatment using all three BeO samples and the respective control groups occurred on the same day. The dishes were cultured until Day 30 and then were fixed, stained, and scored for transformation frequency. For this assay, only foci scored as Class II or III (i.e., having greater than 1300 cells/mm^2) were used to calculate transformation frequencies. A second independent assay was performed two weeks later. A high percentage of cell progeny from such foci have been shown to form malignant tumors when injected into nude mice (Steele et al., 1988a).

An alkaline elution technique was used to measure the ability of the three BeO samples to cause DNA single-strand breaks in the RTE cells. The RTE cells were plated as described above, and the cell culture monolayer was allowed to reach about 80% confluence. The cultures then were exposed for 24 h to the same concentrations of the test materials used in the RTE Transformation Assay. The positive control cultures were treated with 1 mg cadmium sulfate per mL. The treated and control cells then were harvested by trypsinization, centrifuged, and resuspended in phosphate-buffered saline (PBS). The cells then were placed on a 0.2-µm polycarbonate filter, lysed, and eluted under alkaline conditions into ten 90-min fractions. The DNA in the fractions was quantified using a spectrofluorometer and a Hoechst dye technique, using calf thymus DNA to generate a standard curve (Cesarone et al., 1979) DNA that contains more single-strand breaks elutes faster, because it is smaller in size and passes through the filter more easily

The transformation frequencies induced by each test substance were determined by calculating the number of transformed colonies per surviving cell colony at each concentration. The maximum increased elution rate above the rate for control cells was determined for each concentration by subtracting the percent of the treated DNA retained on the filter after each fraction

from the percent of control DNA remaining on the filter after each corresponding fraction The compounds were considered positive based on the following criteria

Transformation – if they increased the mean transformation frequency at least twofold above background levels in both independent assays This criterion was used in a recent study using the same RTE system to identify carcinogens versus noncarcinogens (Steele et al , 1988b). In that study, the RTE assay correctly identified 9 of 10 animal carcinogens and 6 of 7 compounds found not to be carcinogenic in animals.

DNA Damage – if they increased the elution rate by 20% compared to controls in both independent assays Normal variation in the amount of DNA retained on the filter for control cells using the methods described in this report is ~ ± 10% of the mean value, while similar values for carcinogen-treated cells are typically 20% greater (unpublished data). Therefore, ± 20% was chosen *a priori* to be the limit of the control values, and greater values would therefore indicate a positive amount of DNA damage In a study by Petzold and Swenberg (1978), the variation (standard deviation/mean) in values similarly derived for control lung tissue was ± 8%

RESULTS

The Be1 transformation data from the first RTE Transformation Assay are shown in Table 5.3-1. The media control had a background transformation rate of 0.25%. The lowest concentration of Be1 induced less than a 1.2-fold increase in transformation compared to the control, and the higher concentrations had transformation ratios less than 1.0 Because there was less than a twofold increase in the transformation rate at any concentration, Be1 was considered negative in this assay In the second RTE Transformation Assay (Table 5.3-2) using Be1, the media control had a background transformation rate of 0.13% The 3-µg/mL and the 0.3-µg/mL groups had the highest transformation rates at 0.55 and 0.53, respectively, resulting in transformation ratios of 4.3 and 4.2, respectively. However, only a 1.5-fold increase was observed at the 1-µg/mL concentration. The results of the second assay were considered positive However, the criteria for being positive require that both tests be positive, therefore, Be1 was not considered positive. The positive control compound, benzo[a]pyrene (B[a]P), produced transformation rates 50 and 85 times greater than that observed in the control groups in the two assays, respectively.

The low-fired BeO sample, Be2, induced transformation in the first RTE Transformation Assay at a peak rate of 1.2% (at 1 µg/mL) or about fivefold greater than the media control (Table 5 3-1) Three concentrations, 0 3, 1 0, and 3 0 µg/mL, induced a greater than twofold increase compared to controls Therefore, Be2 was considered positive in this test In the second RTE Transformation Assay (Table 5 3-2) using Be2, 0 3 µg/mL induced a transformation rate of 0.46% compared to 0 13% for the control (Table 5 3-2), which represents a 3.6-fold increase in transformation compared to control Therefore, Be2 was considered positive in the second assay Because both assays were positive, Be2 was considered a positive compound in the RTE assay

TABLE 5.3-1. EFFECTS OF BERYLLIUM OXIDE TEST SAMPLES ON THE TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS: ASSAY #1

Test Article	Concentration (µg/mL)	Number of Dishes	CFC[a] per Dish	Std. Dev.	Foci Per Dish[b]	Std. Dev.	Percent Transformation[c]	Std. Dev.	Transformation Ratio[d]
Be1	30.00	18	136	14.1	0.000	0.00	0.000	0.000	0.000
	10.00	20	190	11.7	0.050	0.22	0.026	0.001	0.107
	3.00	20	196	7.8	0.150	0.36	0.076	0.002	0.312
	1.00	20	206	18.3	0.350	0.48	0.170	0.002	0.694
	0.30	20	208	20.6	0.600	0.80	0.289	0.004	1.178
Be2	30.00	17	99	7.9	0.059	0.24	0.059	0.002	0.242
	10.00	16	104	14.2	0.125	0.33	0.120	0.003	0.491
	3.00	16	135	11.0	1.188	1.07	0.880	0.008	3.590
	1.00	17	150	19.8	1.824	1.76	1.217	0.012	4.961
	0.30	19	149	24.0	0.737	0.91	0.493	0.006	2.012
Be3	30.00	19	143	28.2	0.211	0.36	0.148	0.003	0.602
	10.00	19	192	6.5	0.053	0.22	0.027	0.001	0.112
	3.00	19	176	15.4	0.105	0.45	0.060	0.003	0.245
	1.00	20	209	13.3	0.050	0.22	0.024	0.001	0.097
	0.30	19	177	5.6	0.263	0.44	0.148	0.002	0.605
Media	0.00	18	227	7.9	0.556	0.83	0.245	0.004	1.000
Solvent	0.2%	19	229	30.6	0.368	0.48	0.161	0.002	0.655
B[a]P	10.00	20	197	13.3	15.850	3.15	8.064	0.017	50.196

[a] CFC = colony-forming cells or cells that are reproductively intact
[b] Number of transformed foci divided by the number of dishes
[c] Foci per Dish / CFC per dish
[d] Percent transformation (treated)/percent transformation background (control)

TABLE 5.3-2. EFFECTS OF BERYLLIUM OXIDE TEST SAMPLES ON THE TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS: ASSAY #2

Test Article	Concentration (µg/mL)	Number of Dishes	CFC[a] per Dish	Std. Dev.	Foci Per Dish[b]	Std. Dev.	Percent Transformation[c]	Std. Dev.	Transformation Ratio[d]
Be1	30.00	15	11	3.4	0.000	0.00	0.000	0.000	0.000
	10.00	15	165	100.6	0.067	0.25	0.041	0.002	0.317
	3.00	11	264	14.6	1.455	1.44	0.551	0.005	4.303
	1.00	13	273	12.7	0.538	0.63	0.197	0.002	1.543
	0.30	13	245	15.3	1.308	0.91	0.533	0.004	4.165
Be2	30.00	9	87	18.1	0.000	0.00	0.000	0.000	0.000
	10.00	14	195	14.1	0.214	0.56	0.110	0.003	0.860
	3.00	8	223	6.3	0.125	0.33	0.056	0.001	0.438
	1.00	14	225	14.4	0.500	0.73	0.222	0.003	1.737
	0.30	13	218	3.4	1.000	1.11	0.459	0.005	3.588
Be3	30.00	14	202	8.2	0.071	0.26	0.035	0.001	0.276
	10.00	13	265	11.5	0.692	0.98	0.261	0 004	2.039
	3.00	13	281	23.7	1.308	1.43	0.466	0.005	3.642
	1.00	14	248	18.4	1.643	1.80	0.661	0.007	5.167
	0 30	15	247	19.3	1.267	1.39	0.513	0.006	4.007
Media	0.00	12	260	23.1	0.333	0.62	0.128	0.002	1.000
Solvent	0.2%	9	259	17.7	0.222	0.42	0.086	0.002	0.671
B[*a*]P	10 00	9	233	8.9	17.111	5.80	7.340	0.025	85.441

[a] CFC = colony-forming cells or cells that are reproductively intact
[b] Number of transformed foci divided by the number of dishes
[c] Foci per Dish / CFC per dish
[d] Percent transformation (treated)/percent transformation background (control)

In the first RTE Transformation Assay using Be3, the highest transformation rate was 0 15% at 0.3 µg/mL (Table 5.3-1). The control transformation rate was 0.25%. The highest transformation rate ratio observed for Be3 was 0.605 at 0 3 µg/mL. Therefore, the first transformation assay for Be3 was considered negative. In the second RTE Transformation Assay with Be3, the groups exposed to concentrations of 3.0, 1.0, and 0.3 µg/mL had transformation rates anging from 0.47 to 0.66%, resulting in transformation ratios from 3.6 to 5.2 (Table 5 3-2). Therefore, the results of the second transformation assay indicate positive findings for Be3. However, since both transformation assays were not positive, the compound was not positive based on the criteria for the RTE Transformation Assay.

The alkaline elution procedure was used to assess DNA damage in the same cell type used in the transformation assay The cell stocks used in these assays were isolated at the same time as those used in the two transformation assays described above. To represent the results visually, the net change from control elution values (i e., the percent of total DNA from treated cells remaining on the filter minus the value for control cells) was plotted against the fraction number. Figure 5.3-1A shows the results for Be1 in the first assay. The DNA content of fractions at all concentrations was within 20% of control values Therefore, Be1 was considered to have negative DNA damaging potential in the first assay In the second alkaline elution assay, only the lowest concentrations of Be1, 0.3 µg/mL, resulted in the elution of more than 20% of the DNA than in the control cells (Figure 5.3-1B). The values for the first two fractions of the 10 µg Be1/mL group also were greater than ± 20% of control values. Therefore, the second assay was considered positive. The DNA from the positive control, 1 mg cadmium sulfate/mL, eluted 20 to 30% faster than that of untreated controls in both assays. Since both assays for Be1 were not positive, Be1 was not considered positive, based on the evaluation criteria.

The results of the first alkaline elution assay for Be2 are shown in Figure 5.3-2A. The DNA from cells exposed to 1µg Be2/mL eluted 20 to 30% faster than that of the controls in 9 of 10 fractions. In the group exposed to 30 µg Be2/mL, only the first fraction indicated that 20% less DNA remained on the filter than in the control group. In the second alkaline assay of Be2 (Figure 5.3-2B), the DNA from cells exposed to 1 µg/mL again eluted 20 to 40% faster than that of the control cells. Therefore, Be2 exposure indicated DNA damage in both assays sufficient to meet the criteria for a positive response

The DNA damage response of RTE cells exposed to Be3 is shown in Figure 5.3-3A Of the five treatment concentrations of Be3, only values for cells treated with 3 µg/m' were greater or less than 20% of control values. Therefore, the first assay was considered positive. In the second assay, Be3 treatment did not induce adequate amounts of DNA damage to be considered positive (Figure 5 3-3B) Therefore, Be3 was not considered positive for inducing significant DNA damage.

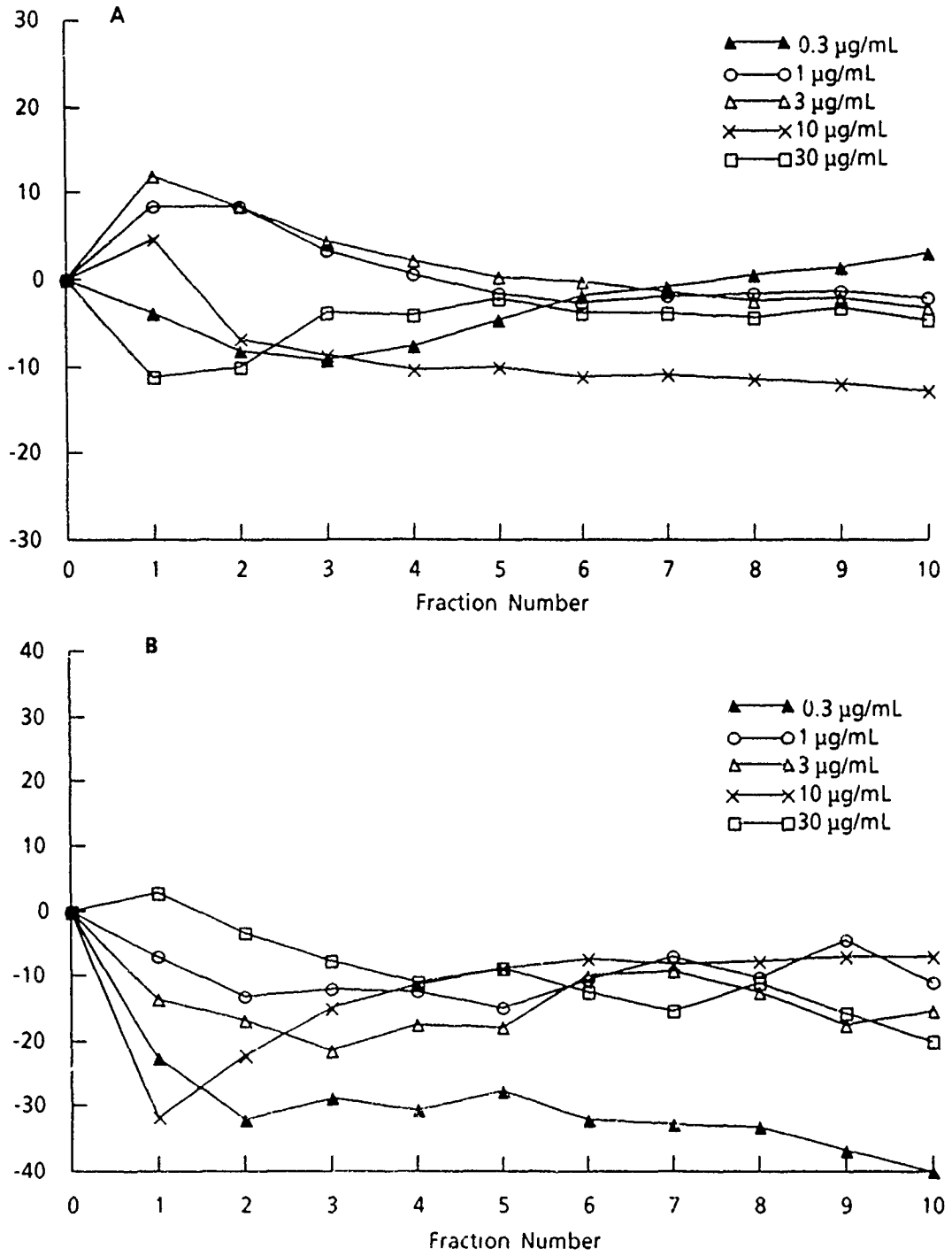

Figure 5.3-1. Effects of Be1 on the Relative Alkaline Elution Rate of Rat Tracheal Epithelial Cell DNA. A = Assay #1, B = Assay #2 Each data point is the difference in the percent total DNA remaining on the filter between treated and control groups and is plotted for each fraction.

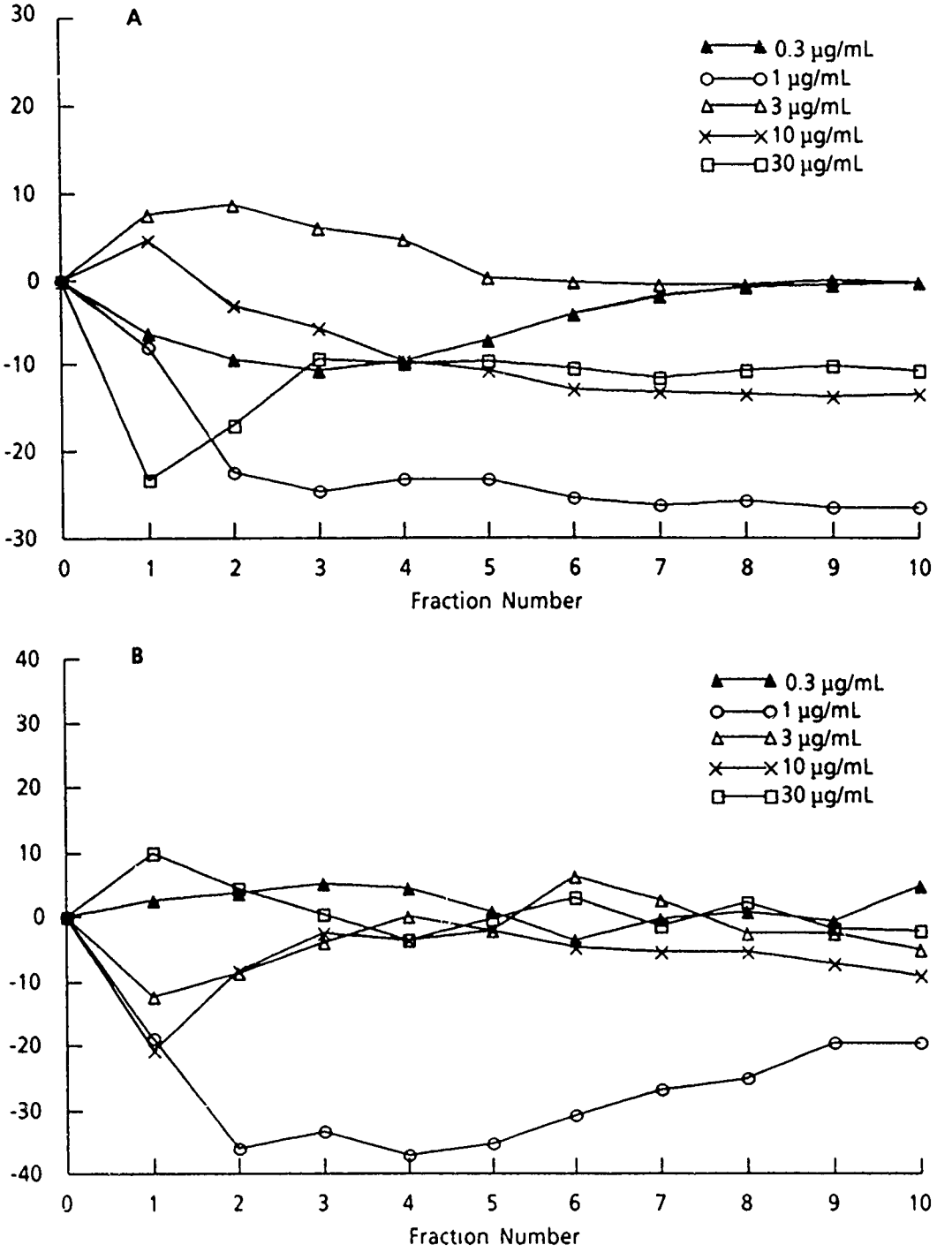

Figure 5.3-2. **Effects of Be2 on the Relative Alkaline Elution Rate of Rat Tracheal Epithelial Cell DNA.** A = Assay #1, B = Assay #2 Each data point is the difference in the percent total DNA remaining on the filter between treated and control groups and is plotted for each fraction

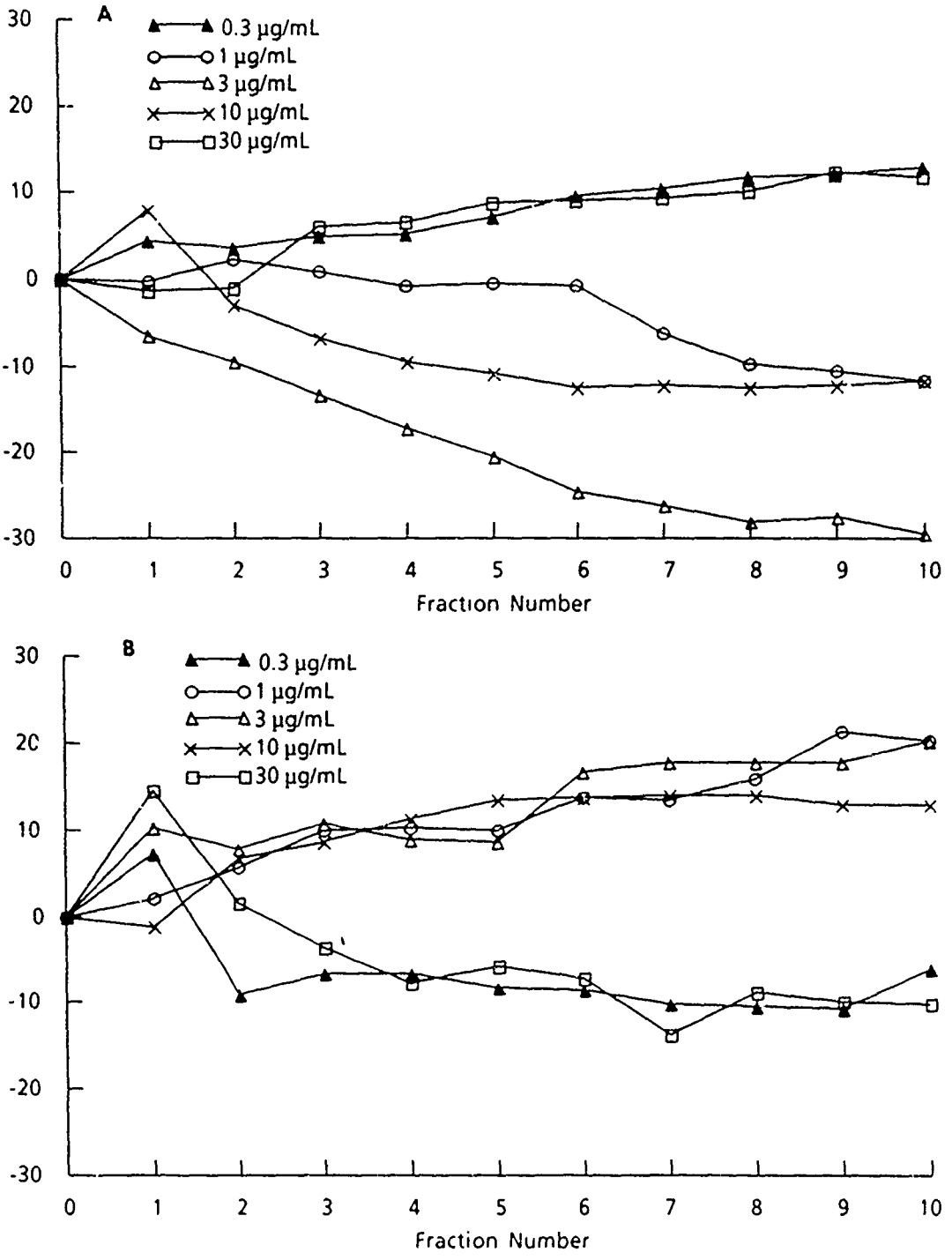

Figure 5.3-3. Effects of Be3 on the Relative Alkaline Elution Rate of Rat Tracheal Epithelial Cell DNA. A = Assay #1, B = Assay #2 Each data point is the difference in the percent total DNA remaining on the filter between treated and control groups and is plotted for each fraction

DISCUSSION

Previous cytotoxicity studies (Steele and Kutzman, 1988) indicated that the BeO particles available for these investigations were toxic to cultured respiratory epithelial cells at concentrations above 10 to 30 µg/mL. Based on those results, the BeO samples were tested for the ability to both transform and induce DNA damage in RTE cells in the range of 0.3 to 20 µg/mL.

The results of the RTE Transformation Assay and the RTE DNA Damage Assay are summarized in Table 5.3-3. The Overall Response column indicates a positive result if both assays were positive, an equivocal result if only one assay was positive, and a negative result if both assays were negative. Previous experience with these assays has shown that carcinogens (e.g., the positive controls) typically give positive results in replicate assays. These assays, as well as all other short-term tests, are not perfect in predicting animal carcinogenicity. Using criteria similar to those used in this report for a positive result, the RTE Transformation Assay correctly identified 9 of 10 animal carcinogens (one false negative) and 6 of 7 noncarcinogens in a previous study (Steele et al., 1988b). In previous experience with the alkaline elution assay, the DNA from cadmium-exposed cells (using a concentration that transforms RTE cells) nearly always eluted at a rate at least 20% greater than that of the controls, and these results were repeatable in duplicate experiments (unpublished data).

TABLE 5.3-3. SUMMARY TABLE OF THE EFFECTS OF THE BERYLLIUM OXIDE TEST SAMPLES ON RAT TRACHEAL EPITHELIAL CELLS

	Transformation Assay		
Test Material	Assay #1	Assay #2	Overall Response
Be1	–	+	±
Be2	+	+	+
Be3	–	+	±
DMSO	–	–	–
B[a]P	+	+	+

	DNA Damage Assay		
Test Material	Assay #1	Assay #2	Overall Response
Be1	–	+	±
Be2	+	+	+
Be3	+	–	±
Medium	–	–	–
$CdSO_4$	+	+	+

The results of the RTE DNA Damage Assay paralleled those of the RTE Transformation Assay in that Be2 was positive while Be1 and Be3 were not. In both assays, the maximum amount of DNA damage was incurred by exposing the cells to 1 µg/mL of the low-fired BeO, Be2. Higher concentrations of Be2 may have induced DNA-DNA crosslinking and therefore would appear to have fewer single-strand breaks (Sunderman, 1984). The damage observed at 1 µg Be2/mL was equal to or greater than the damage observed following treatment with 1 mg cadmium sulfate/mL, another DNA-damaging agent. These results indicate that BeO exhibits a 1000-fold greater potency than cadmium in terms of DNA-damaging ability. However, the concentration of cadmium required to transform RTE cells is of the same order of magnitude as that needed by the BeO, Be2 (Steele et al., 1988b).

Be1, the rocket test sample, and Be3, the high-fired BeO sample, did not meet the criteria for positive tests in either the RTE Transformation or the DNA Damage bioassays. These results may be somewhat equivocal because findings were inconsistent between the independent assays (Table 5.3 3) However, these materials appear to be less biologically active than the Be2, which produced uniformly positive results in both tests of the Transformation Assay and the DNA Damage Assay. The general concordance of results between these assays supports the consensus that DNA damage plays a key role in the transformation of mammalian cells (for review, see Harper and Morris, 1984) These studies also support the previous findings of Spencer et al., (1968) in which low-fired BeO particles were found to be more toxic than high-fired BeO particles. In comparison with previous RTE test results, the low-fired BeO sample, Be2, induced transformation ratios comparable to cadmium sulfate, 4,4'-methylene bis(2-chloroaniline), or di(2-ethylhexyl)phthalate, but lower than the polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene or B[*a*]P (Steele et al , 1988b).

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SECTION 6

PHARMACOKINETIC AND PHARMACODYNAMIC MODELING

6.1 GASTROINTESTINAL ABSORPTION OF XENOBIOTICS IN PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS: A TWO-COMPARTMENT DESCRIPTION

D.A. Staats and R.B. Conolly

INTRODUCTION

The Installation Restoration Program (IRP) has identified numerous chemical contaminants in groundwater Many of these chemicals have been shown to be carcinogenic in National Institutes of Health bioassays Human exposure to these contaminants occurs by ingestion of drinking water derived from this groundwater. However, corn oil rather than water was used as the dosing vehicle in these studies Profound effects of vehicle on chemical absorption by the gastrointestinal tract have been demonstrated (Chieco et al., 1981; Withey et al., 1983). Therefore, proper assessment of carcinogenic risk from drinking water exposure requires an understanding of the effect of various vehicles on the rate of gastrointestinal absorption

One tool for assessing risk resulting from gastrointestinal absorption of a toxicant is physiologically based pharmacokinetic (PB-PK) modeling. When using this approach, absorption of a chemical from the gastrointestinal tract typically has been described as a one-compartment, first-order process Although this adequately simulates the appearance of chemical in the blood when the compound is administered in water, it poorly describes absorption from an oily medium

In this study, a two-compartment description was developed in which absorption occurred via first-order processes from both the first and second compartments Transfer from the first to the second compartment was described also as a first-order process Blood concentration data after oral gavage of methylene chloride (M-CHL), chloroform (CHLF), or dichloroethane (DCE), in corn oil or water, or trichloroethylene (TCE) in water were used for model validation (Withey et al , 1983, Withey, 1985 personal communication).

MATERIALS AND METHODS

Data Base

Withey et al (1983) published blood concentration-time curves obtained in male Wistar rats (average body weight 400 g) following oral gavage of one of four halogenated hydrocarbons Animals were dosed with 3 to 5 mL of M-CHL (125 mg/kg), CHLF (75 mg/kg), or DCE (100 mg/kg) in water or corn oil, or TCE (18 mg/kg) in water Blood samples were taken at 2, 4, 6, 10, 13, 16, 19, 22,

25, 30, 40, 60 min and every half hour thereafter until 5 h post-dosing. Blood samples were analyzed by a head-space gas chromatographic technique (Withey and Collins, 1980)

Physiologically Based Pharmacokinetic Modeling

PB-PK models consist of physiologically realistic compartments Mass balance differential equations for each compartment describe the rate of change in the amount of chemical. A PB-PK model based on Ramsey and Andersen (1984) and Andersen et al (1987) with a one-compartment, first-order description of gastrointestinal absorption was modified for use in the present study. In this modification, a two-compartment description of gastrointestinal absorption was developed in which the dose moves from the first to the second compartment (first-order rate constant, KT) and is absorbed from both compartments (first-order rate constants KAS and KAD, respectively). A schematic diagram representing this description of gastrointestinal absorption is shown in Figure 6.1-1 KAS, KAD, and KT in the two-compartment description of absorption were optimized with Simusolv (Dow Chemical Co , Midland, MI) to obtain the best fit of the blood concentration time course data for each dosing solution. KAD and KT in the one-compartment description of absorption were set to zero and only KAS was optimized with Simusolv to obtain the best fit of the data set for each dosing solution. Parameters in the model such as molecular weight, V_{max}, K_m, and tissue partition coefficients (Gargas et al., 1988a, 1988b) were set to those specific for the chemical in the dosing solution.

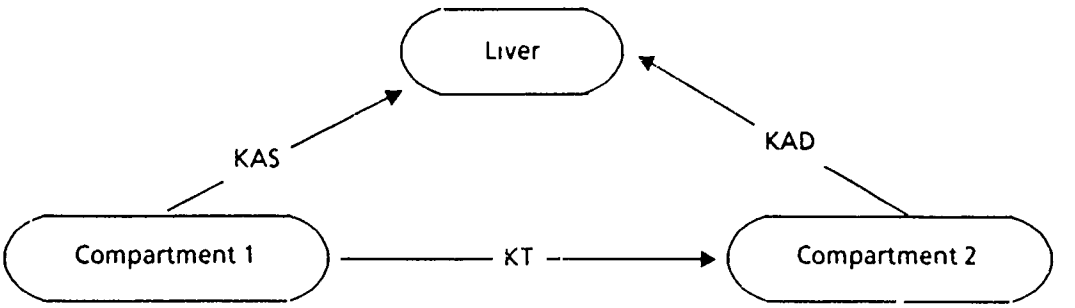

Figure 6.1-1. Schematic Representation of the Two-Compartment Description of Gastrointestinal Absorption.

Computer Hardware and Software

The PB-PK model was written in the FORTRAN-based Advanced Continuous Simulation Language (ACSL) (Mitchell & Gauthier Associates, Inc , Concord, MA) Simusolv (Dow Chemical Co , Midland, MI), an enhanced version of ACSL with optimization capabilities, was used to run the program on a VAX 8530 (Digital Equipment Corp , Maynard, MA)

RESULTS

Figure 6 1-2A depicts the Withey et al (1983) blood concentration-time course data after oral gavage dosing of CHLF (75 mg/kg) in water. Curve b is the simulation of these data using the two-compartment description of gastrointestinal absorption Optimal fit of these data was achieved by setting the first-order rate constant for absorption from the first compartment (KAS = 4.0), the rate constant for absorption from the second compartment (KAD = 0 24), and the first-order rate constant for transfer from the first to the second compartment (KT = 5.0). Curve a is the simulation of these data using a one-compartment description of absorption which is obtained by setting KT = 0. With KT = 0, Simusolv optimization set KAS = 3. However, with this value, the simulation overestimates blood CHLF concentration over time This overestimation is even more severe when the same dose of CHLF is administered in corn oil as shown in Figure 6 1-2B. In this case, simulation using the one-compartment description (Curve a) overestimates the data Although the peak blood concentration of CHLF can be simulated by optimizing KAS with Simusolv, the time taken to reach the peak was much greater in the simulation than in the data set Curve b is the simulation of these data using the two-compartment model. The optimal KAS, KAD, and KT values determined by Simusolv are listed in Table 6 1-1.

The same trends are seen in the simulations of the blood concentration-time course data after oral dosing of M-CHL (125 mg/kg) in water (Figure 6 1-3A) or in corn oil (Figure 6.1-3B). Model parameters described above were set to values specific for M-CHL (Gargas et al., 1988a and 1988b). The data set in Figure 6.1-3A was simulated accurately (Curve b) by optimizing KAS, KAD, and KT in the two-compartment description, and adequately simulated by setting KAS = 6.0 in the one-compartment description (Curve a) Curve a only slightly overestimates the blood M-CHL concentration over time However, the corn oil data in Figure 6 1-3B are highly overestimated by the one-compartment description (Curve a) (KAS = 0 9) The data were simulated adequately by optimizing KAS = 1 7, KAD = 0 3, and KT = 2 6 in the two-compartment (Curve b) description of gastrointestinal absorption

Blood DCE concentration time course data after oral gavage (100 mg/kg) in water (Figure 6 1-4A) and in corn oil (Figure 6 1-4B) were also used for model validation. Again, the two-compartment description (Curve b) simulated both data sets adequately (see Table 6 1-1 for constant values) The one-compartment description resulted in overestimation of the data (Curve A), particularly in Figure 6 1-4B (corn oil vehicle) In addition, the time to peak blood DCE concentration was overestimated in the simulation in Figure 6 1-4B

Blood concentration time course data (Figure 6 1-5) after oral gavage of TCE in water (18 mg/kg) were simulated using the one (Curve a) and the two-compartment (Curve b) description of absorption Curve a is an adequate simulation, however, Curve b depicts an even more accurate fit of the data set

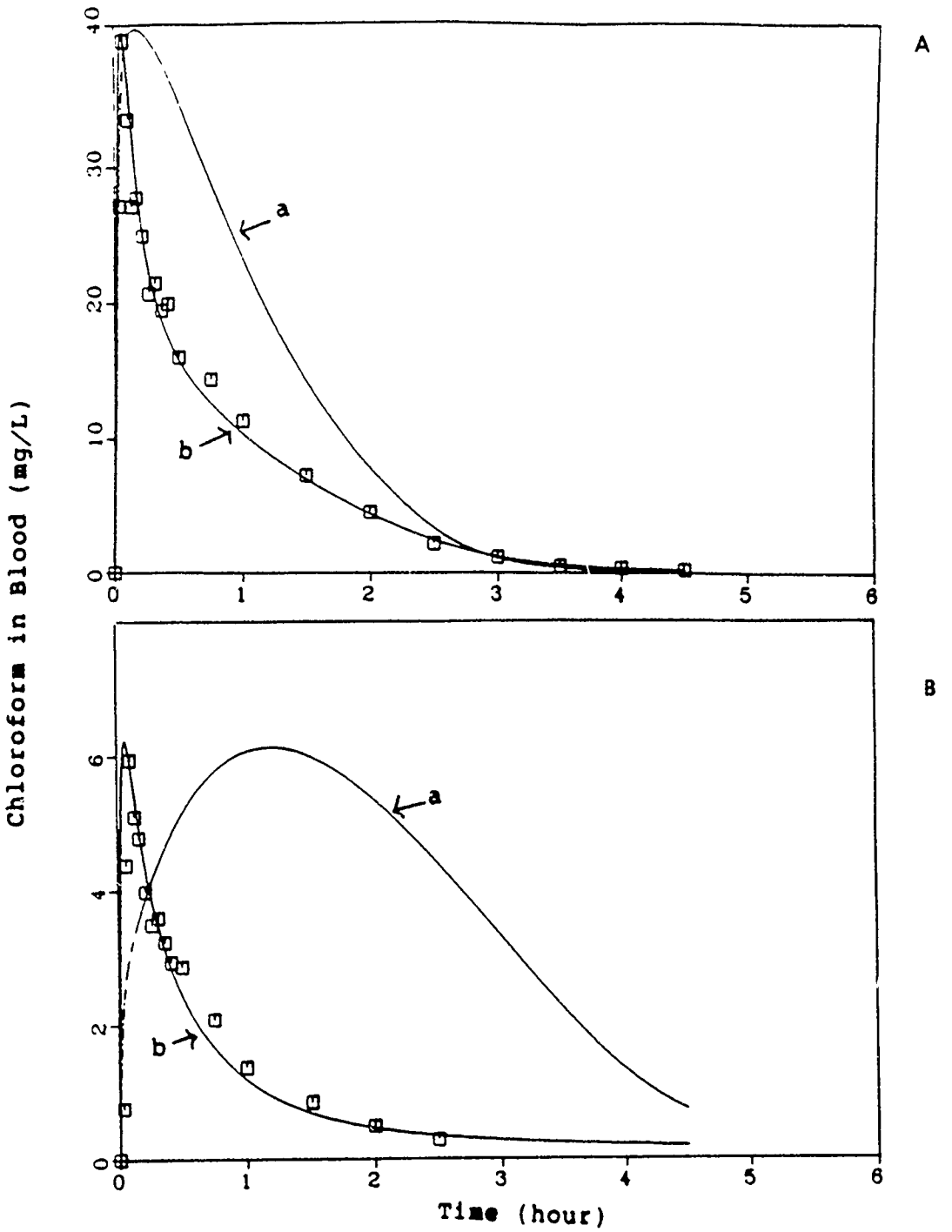

Figure 6.1-2. Venous Concentration of Chloroform after Oral Gavage Dosing (75 mg/kg) in Water (A) or in Corn Oil (B). Data set was simulated using one-(curve a) or two-compartment (curve b) description of gastrointestinal absorption.

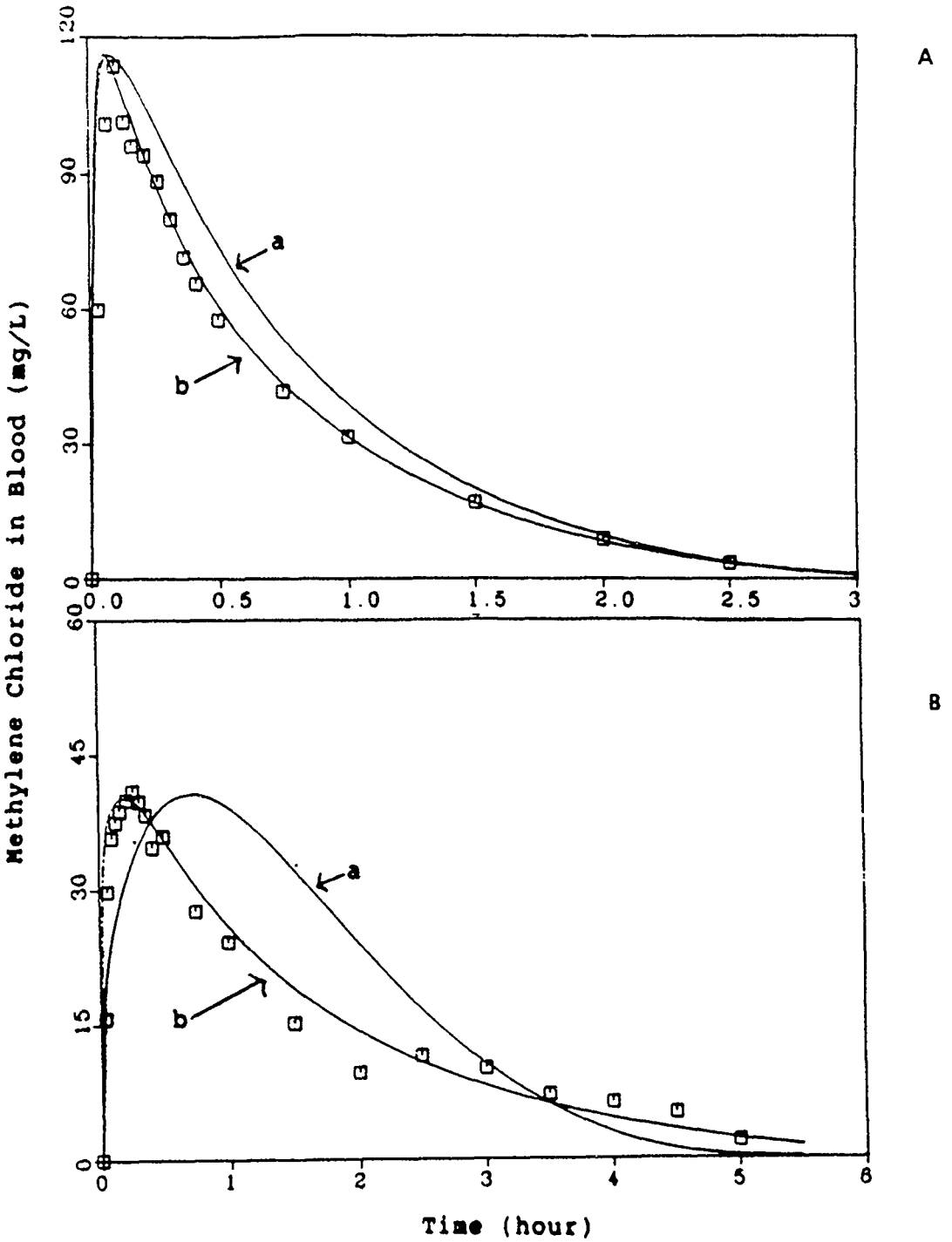

Figure 6.1-3. Venous Concentration of Methylene Chloride after Oral Gavage Dosing (125 mg/kg) in Water (A) or in Corn Oil (B). Data set was simulated using one-(curve a) or two-compartment (curve b) description of gastrointestinal absorption

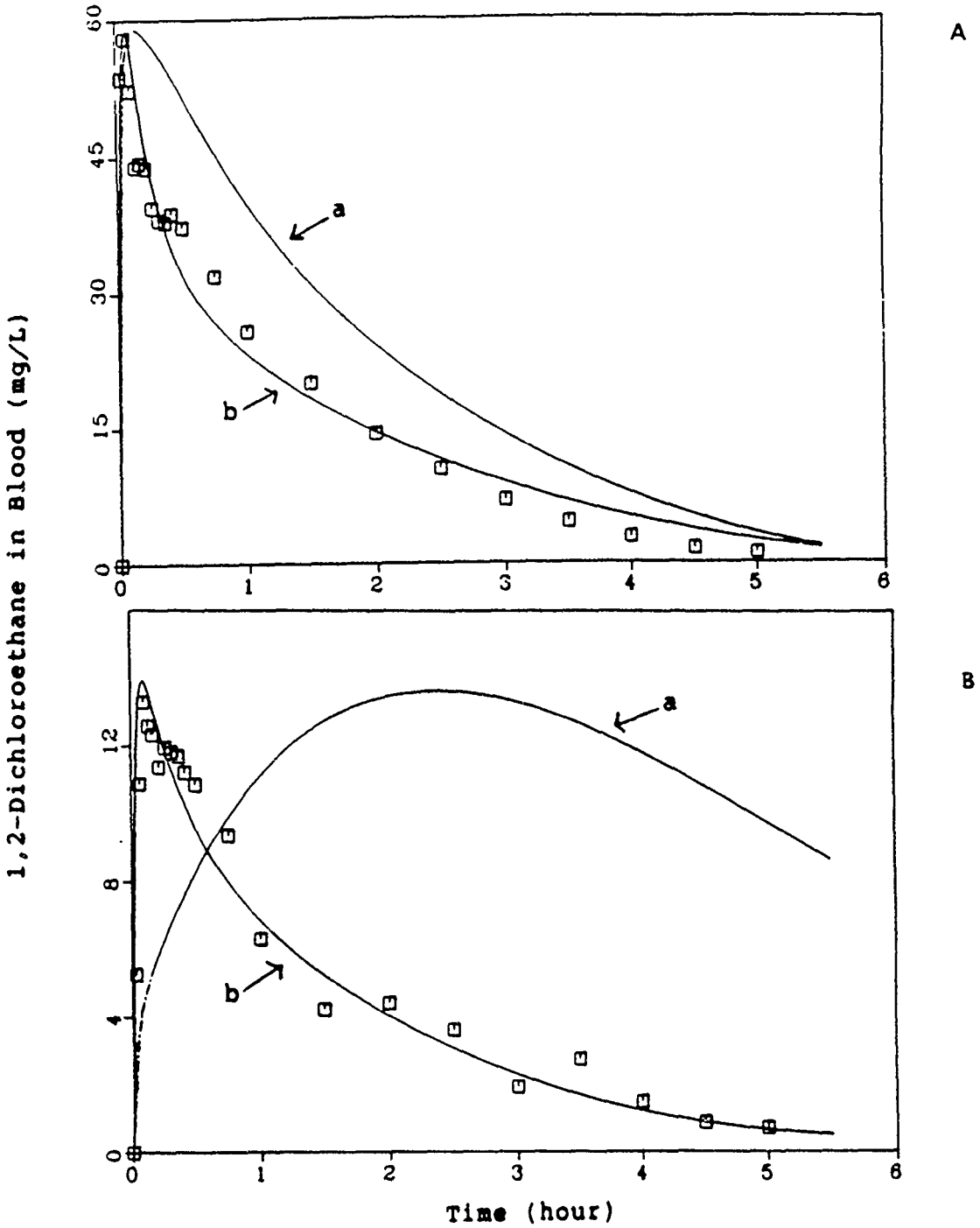

Figure 6.1-4. Venous Concentration of 1,2-Dichloroethane after Oral Gavage Dosing (100 mg/kg) in Water (A) or in Corn Oil (B). Data set was simulated using one-(curve a) or two-compartment (curve b) description of gastrointestinal absorption.

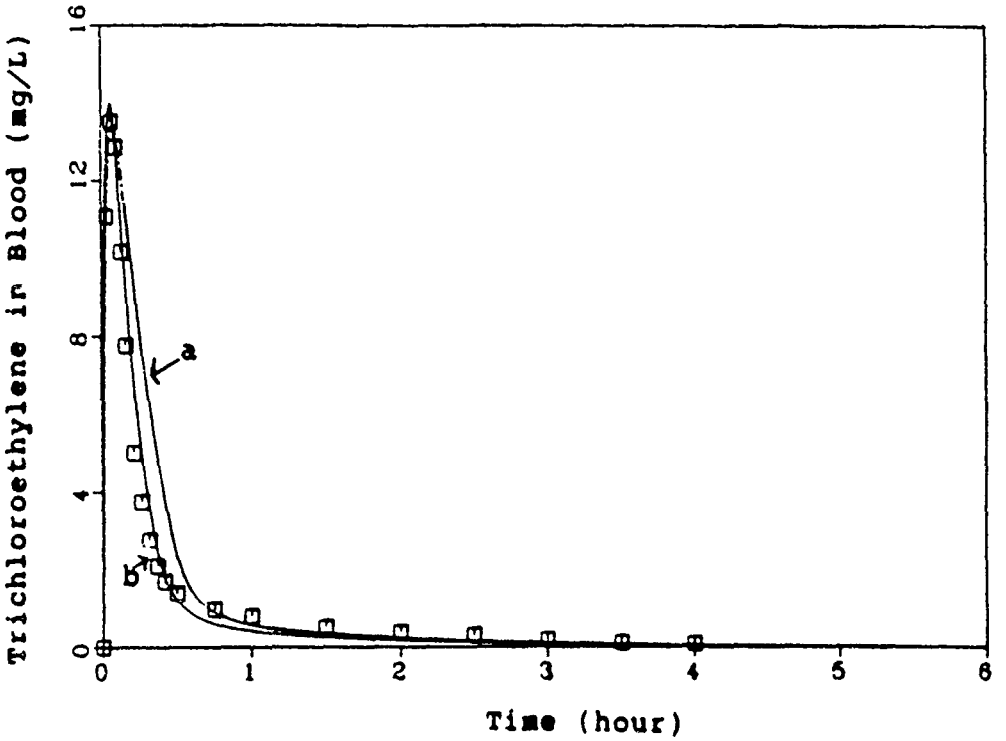

Figure 6.1-5. Venous Concentration of Trichloroethylene after Oral Gavage Dosing (18 mg/kg) in Water. Data set was simulated using one-(curve a) or two-compartment (curve b) description of gastrointestinal absorption.

The values of KAS, KAD, and KT used to optimize simulation of blood concentration time course data for each dosing solution are listed in Table 6.1-1. KAS values in simulations of water vehicle data sets were several-fold higher than those used to simulate comparable corn oil vehicle data sets in both one- and two-compartment descriptions In the water vehicle data sets, the KAS values were similar between the one- and two-compartment descriptions for absorption. In contrast, the two-compartment KAS values were approximately twice those used in the one-compartment description simulations for each corn oil vehicle data set. The KAD and the KT values were similar between corn oil and water vehicle data sets in the two-compartment description.

TABLE 6.1-1. UPTAKE AND TRANSFER CONSTANTS

	Two-Compartment Model						
	CHLF		M-CHL		DCE		TCE
	Oil	Water	Oil	Water	Oil	Water	Water
KAS	0 7	4.0	1.7	6.0	0 9	4 0	7.0
KAD	0.1	0 24	0.3	0 1	0 04	0 01	0 1
KT	6 2	5 0	2 6	1 1	3 9	2 0	2 0
	One-Compartment Model						
KAS	0 31	3.0	0 9	5 6	0 25	3 3	6 0

DISCUSSION

A major goal of the IRP program is reduction of concentrations of groundwater contaminants to acceptable levels. One major difficulty in assessing carcinogenic risk for humans exposed to these chemicals through drinking water is that few toxicity studies have been conducted in which these insoluble hydrophobic compounds were administered in water. In most studies using the oral route of exposure, oily vehicles were used. The extent and rate of absorption of substances from the gastrointestinal tract are affected by the vehicle Withey et al (1983) noted that dosing with a corn oil solution compared to an aqueous solution resulted in lower peak blood concentrations of the compound and an increase in the time necessary to reach peak blood levels. This effect of vehicle on target tissue levels of the toxicant ultimately could affect the outcome of toxicity studies, and therefore must be considered when assessing risk.

PB-PK modeling provides information that can be used in the risk assessment process. However, it is necessary to have a model that can simulate tissue concentration time course data after gastrointestinal absorption of chemical from oily or aqueous vehicles. Previously, a one-compartment description for gastrointestinal absorption was used This approach is adequate for water but not for oily vehicles The two-compartment approach presented herein describes gastrointestinal absorption accurately for both aqueous and corn oil vehicles.

In all cases studied, the two-compartment description simulated blood concentration time course data more accurately than the one-compartment description. With any chemical studied, the first-order rate constant for absorption from the first compartment, KAS, was far higher when water rather than corn oil vehicle was used. This indicates that absorption from the first compartment (probably the stomach) is enhanced when fat soluble toxicants are administered in water. We speculate that when administered in corn oil, these toxicants prefer the lipid environment of the vehicle to the aqueous environment of the stomach, and therefore, absorption of the chemical is retarded. With all dosing solutions the first-order rate constant for absorption from the second compartment (KAD) was very low compared to the KAS This suggests that the rate of absorption from the second compartment (probably the small intestine) is slow KT, the first-order rate constant for transfer from the first to the second compartment, was higher when the chemical was administered in oil rather than in water It is well established that absorption of oils takes place in the small intestine, not the stomach

In a daily dosing scenario, the model predicted a slight increase in the peak amount of chemical in the second compartment after the first day This increase was due to a small amount of the dose remaining in the second compartment 24 h after the first dose However, equilibrium between absorption and elimination was reached within two to three days

Experiments are being designed to explore the physiological relevance of the rate constants for absorption and transfer Until a physiologic model is developed, this simple two-compartment approach can be used to describe gastrointestinal absorption from both water and oily vehicles

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SECTION 7

AIR FORCE CHEMICAL DEFENSE TOXICOLOGY

7.1 A PHYSIOLOGICALLY BASED PHARMACODYNAMIC MODEL FOR INHIBITION OF ACETYLCHOLINESTERASE BY DIISOPROPYLFLUOROPHOSPHATE

J.M. Gearhart, G. Jepson[a], H.J. Clewell, III[a], M.E. Andersen[a], and R.B. Conolly

INTRODUCTION

In combat situations, personnel may be exposed to organophosphorus nerve agents, resulting in diminished performance of pilots operating high-performance aircraft and health risks to medical personnel in field hospitals. To provide medical treatment, determine protective posture, and plan effectively for a battle scenario where there is potential for chemical warfare, it is necessary to obtain estimates of personnel exposure and predict their possible incapacitation. One of the greatest concerns is not the high-level lethal exposures more commonly considered in the past, but the effect of repeated low-level nonlethal exposures.

One method of making more informed estimates of these potential organophosphate (OP) exposure effects is to employ biologically structured models of OP toxicity. These models link what is known about OP pharmacokinetics with the dynamic effects of the compound, specifically acetylcholinesterase (AChE) inhibition. This report describes a physiologically based pharmacokinetic (PB-PK) and pharmacodynamic model (OPM) for the *in vivo* behavior of OPs. It should be useful for risk assessment and management of exposures in military situations. The model is able to simulate the time course of the OP diisopropylfluorophosphate (DFP) in blood, brain, and other organs, and then use this information to calculate the inhibition of OP-susceptible esterases, such as AChE, butyrylcholinesterase (BuChE), and carboxylesterase (CaE). AChE inhibition is especially significant as the target enzyme of acute OP exposure, while BuChE and CaE provide sites of OP loss without deleterious effects.

MATERIALS AND METHODS

OPM Structure

The OPM is structured to link the time course of DFP in different tissue compartments with enzyme inhibition. The amount of residual AChE activity remaining after inhibition is then

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supplemented by synthesis of new enzyme so that AChE activity returns to normal levels The following are the three main aspects of the model.

1. A PB-PK model to describe tissue concentrations of DFP in the different compartments

2. A dynamic effects section using OP specific rate constants to describe the inactivation of AChE. These are the bimolecular rate constants of inhibition, enzyme reactivation, and aging of the enzyme-DFP complex

3. The resynthesis of AChE

DFP Pharmacokinetics

PB-PK models have been developed that track the concentration of toxicants in the different organ compartments (Ramsey and Andersen, 1984; Lutz et al., 1977) These models use the blood flows, organ volumes, chemical-specific affinity, and biotransformation rate for each tissue to determine the absorption, distribution, and elimination of the chemical. The two major model parameters that determine the kinetics of OPs in the PB-PK models are tissue/blood partition coefficients and the metabolic capacity of the organism. Tissue partition coefficients for DFP were determined by the vial equilibration methods of Sato and Nakajima (1979), as previously described (Cramer et al., 1988)

Unlike many other compounds, the largest amount of OP metabolism takes place in the blood by esterases OP pharmacokinetics are strongly affected by reaction with "A" (AEST) and "B" esterases (BEST) BEST are those enzymes having a serine residue at the site of binding that is inactivated by OPs These are primarily AChE, BuChE, and CaE. AEST can hydrolyze OPs without being inhibited This enzyme is present in virtually all tissues, but usually has its greatest activity in the blood As a result, the AEST activity in the blood strongly effects the amount of OP that reaches the AChE in the different target tissues One source of the wide species variation in OP response is the diversity in AEST concentration in the different tissue compartments, especially blood The differential equation for the change in DFP concentration with time due to AEST activity is as follows.

$$d[DFP]/dt = (V_{max} \times CV_L) / (K_m + CV_L)$$

where K_m is the Michaelis constant for AEST oxidation, V_{max} is the maximum velocity of the enzyme reaction, and CV_L is the venous concentration of DFP for the particular organ the equation is describing, in this example the liver

DFP Pharmacodynamics

The model calculates the change in AChE inhibition with time by the following equation

$$d[AChE]/dt = -k_i \times [AChE] \times [DFP]$$

where k_i is the bimolecular rate constant for DFP and the AEST of interest, AChE is the AChE activity in the organ of interest, and DFP is the concentration of DFP in that organ The bimolecular rate constants for the DFP reaction with rat AChE, BuChE, and CaE were determined by Jepson (1986). These same rate constants were used for those simulations of the inhibition of mouse AChE.

AChE Resynthesis

The synthesis of AChE is described as a zero-order process, while the normal degradation follows first-order kinetics (Segel, 1976). The AChE degradation rate for rat brain was taken from literature (Wenthold et al , 1974). The rates of enzyme synthesis were determined by fitting the model to the data for AChE recovery after DFP inhibition. The differential equation for the change in AChE activity with time is as follows:

$$d[AChE]/dt = k_{syn} - k_i \times [AChE] \times [DFP] - k_{deg} \times [AChE]$$

where k_{syn} is the zero-order rate of AChE synthesis and k_{deg} is the first-order rate of AChE degradation.

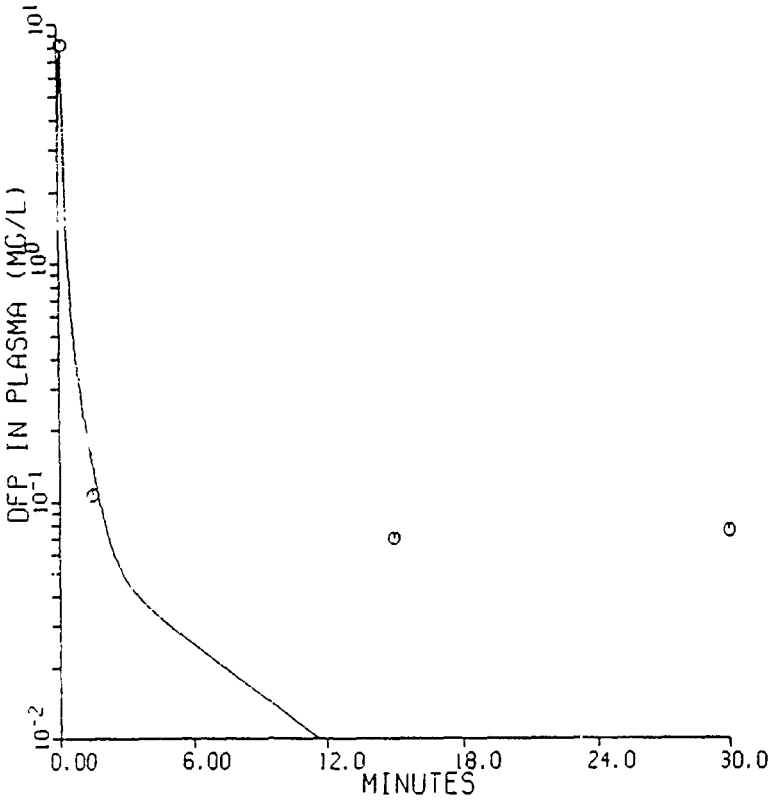
RESULTS

OPM Validation

The ability of the OPM to predict the concentration of DFP in different tissues was modeled for two exposure routes, intravenous (iv) infusion and inhalation exposure

Iv DFP Kinetics and Dynamics in Mice

Martin (1985) injected mice via the tail vein with 1 mg [^{3}H]DFP/kg After exposure, animals were sacrificed at 15 s, 1 min, 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 1 day, 3 days, and 7 days, and free DFP, bound DFP, and the products of DFP metabolism were quantitated in the different tissues. Model predictions of the concentration of free DFP in plasma and brain, and the experimental values are shown in Figure 7 1-1 for the first 30 min post-injection The model predictions are good for both organs through the 1 min time point and then the model predicts a greater disappearance of DFP than was measured experimentally The amount of DFP in plasma at 1 min after injection is only about 1% of the peak initial activity, so that any differences between the predictions and experimental results probably have little bearing on the dynamic effects Two possible explanations for the higher than predicted experimental values are (1) during DFP aging of AChE, a ^{3}H-labeled isopropyl group on DFP will be released and freely extracted with the DFP, giving artificially high free DFP values, and (2) there can be an exchange between the ^{3}H and normal hydrogen that is bound to different tissue components, increasing the amount of ^{3}H extracted with the free DFP



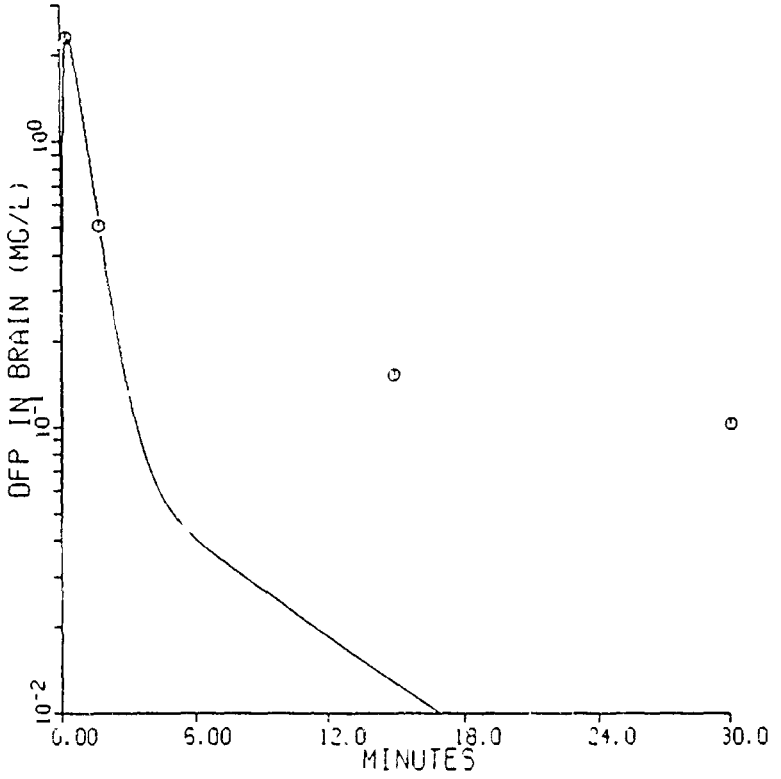


Figure 7.1-1 A & B. Time-Course of DFP Concentration in Plasma and Brain Tissue of Mice Injected iv via the Tail Vein with 1 mg DFP/kg.

The model predictions of AChE inhibition in plasma and brain for 1 h and 24 h are depicted in Figures 7.1-2 and 7.1-3. The simulation of plasma AChE inhibition during the 30 min after injection of DFP was a good prediction of the experimental values, but not as good at 1 and 4 h. This difference most likely is due to experimental variability, since the data for AChE inhibition at 8 and 24 h were closer to model predictions. The simulation of brain AChE inhibition was in good agreement with experimental data (Figure 7.1-3) The simulation of brain AChE resynthesis beyond the 24 h time period (Figure not shown) returned to 100% of control levels, as would be expected, but the experimental values remained at 80% of control for the rest of the 7 days of the study.

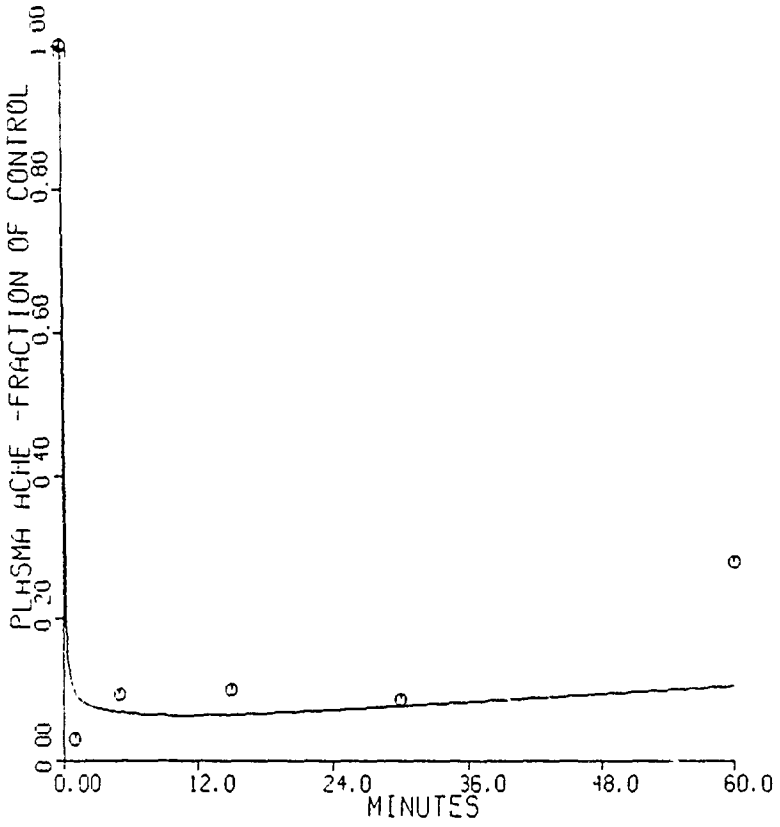
Inhalation of DFP - Kinetics and Dynamics in Mice

Scimeca et al. (1985) exposed mice to DFP vapors for 5 min at 60 ppm and determined the different tissue concentrations of DFP at 5 min, 15 min, 30 min, 1 h, 4 h, 8 h, and 1 day after exposure. Plasma, brain, lung, and diaphragm were assayed for AChE activity at 5 min, 30 min, 60 min, 4 h, 12 h, 1 day, 3 days, and 7 days. The model predictions for plasma and brain concentrations are displayed in Figure 7.1-4. Concentrations of DFP in the different tissues were not determined experimentally during the 5-min inhalation exposure The model predictions of DFP concentrations at the end of the inhalation exposure pass through the experimentally determined values for both plasma and brain. As with the iv injection study, the model describes an almost total clearance of DFP from plasma and brain tissue at an earlier time than the experimental data The reasons for this discrepancy have been discussed in the section describing iv DFP exposure

Predictions of plasma and brain AChE inhibition over the 24-h period were not as close to the experimental values after inhalation as after iv administration (Figures 7 1-5 and 7.1-6) In these simulations, both the plasma and brain AChE activities were inhibited to a greater extent than the experimental data indicated One explanation for this discrepancy is that the interanimal variation in AChE inhibition was much greater for inhalation exposure vs iv infusion Inhalation of a material as toxic as DFP for 5 min could depress respiration and alter the amount of DFP inhaled toward the end of the exposure Motor coordination of DFP-exposed mice was only 35% that of their control and could explain differences between the predicted and measured concentration of DFP and enzyme activity.

Inhibition of Rat Brain and Plasma AChE after Repeated DFP Exposure

To determine the model's ability to predict the effects of repeated DFP exposures, rat studies were simulated in which a subcutaneous (sc) dose of DFP was administered every 48 h, and at 1.5 and 24 h after dosing, animals were sacrificed and the plasma and brain AChE activity was assayed Two different studies from the same laboratory were performed according to this protocol



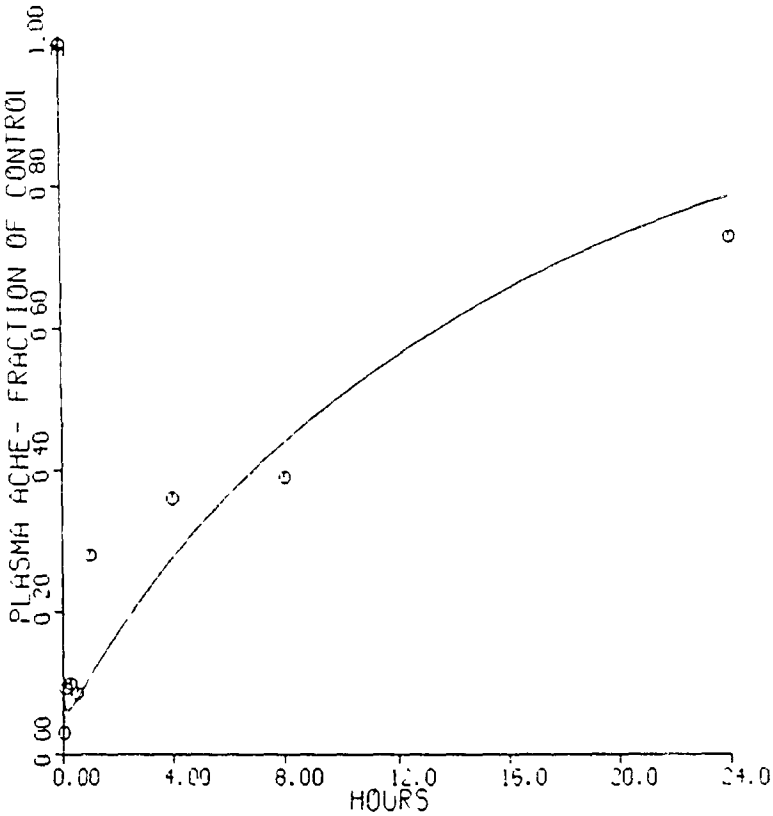


Figure 7.1-2 A & B. Time-Course of Plasma AChE Activity in Mice after 1 mg DFP/kg iv, Expressed as a Fraction of Control Levels of AChE.

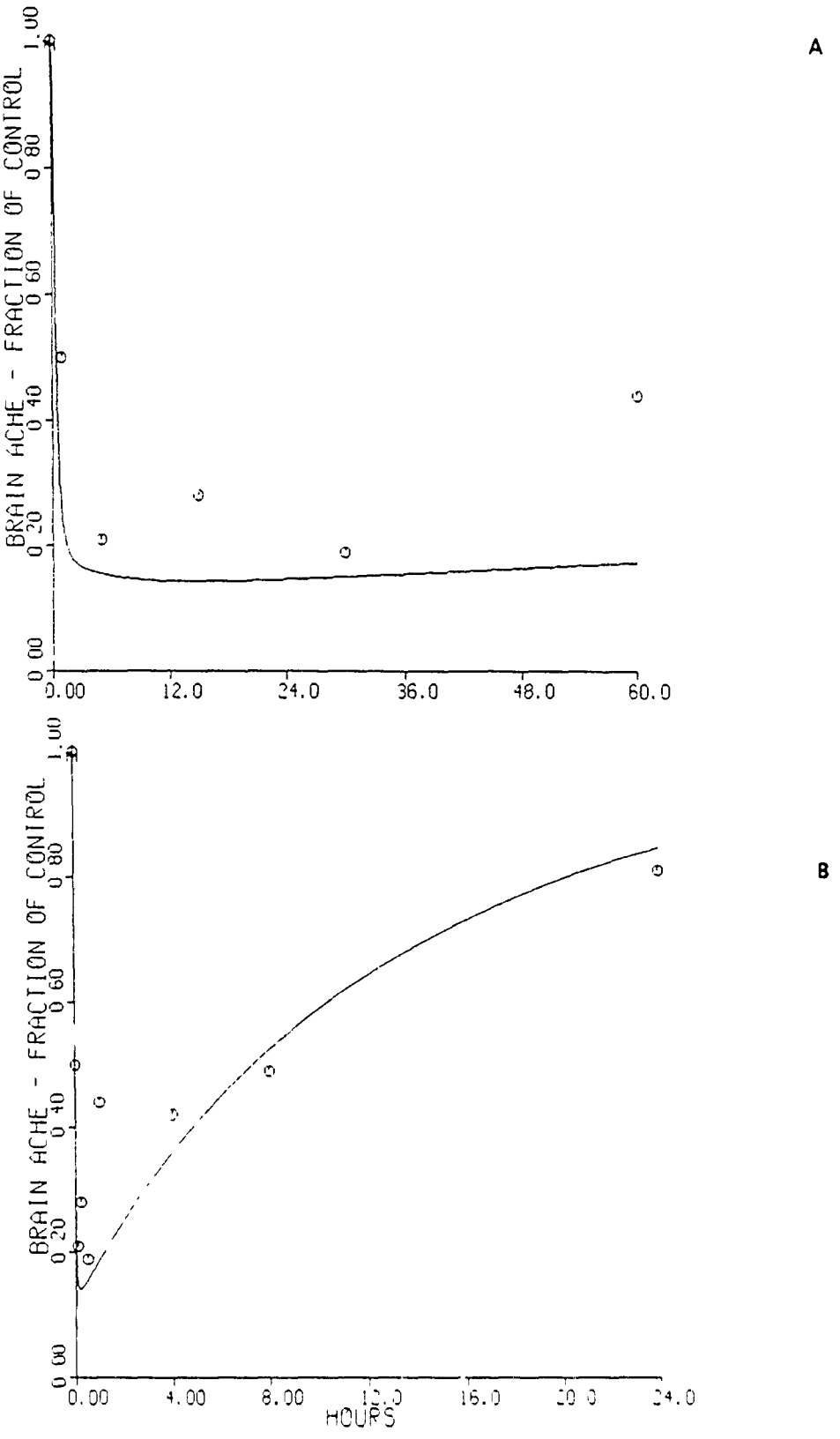
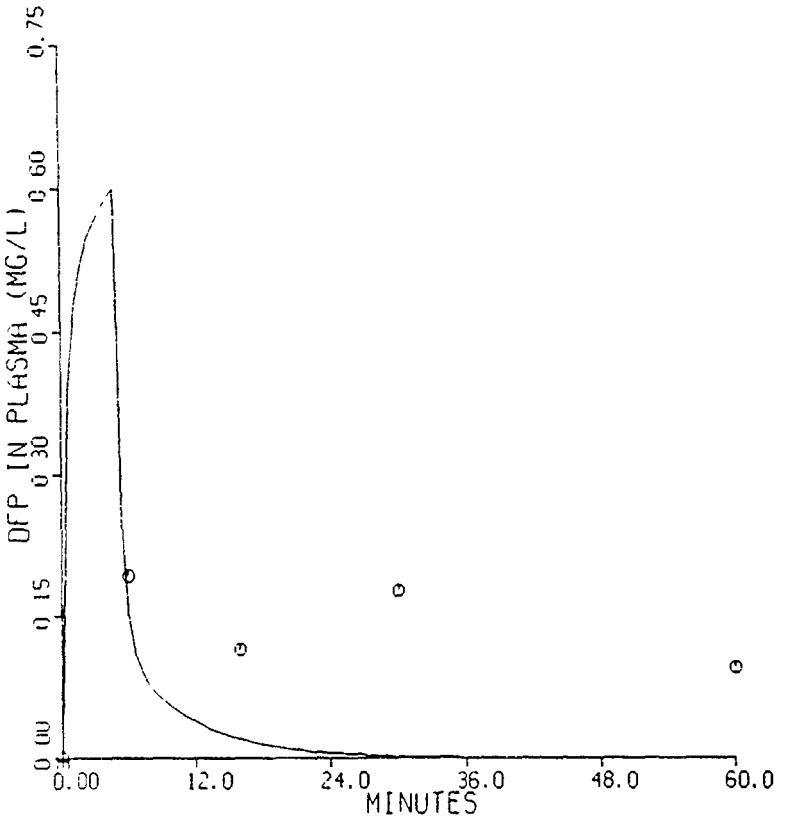


Figure 7.1-3 A & B. Time-Course of Brain AChE Activity in Mice after 1 mg DFP/kg iv, Expressed as a Fraction of Control Levels of AChE.



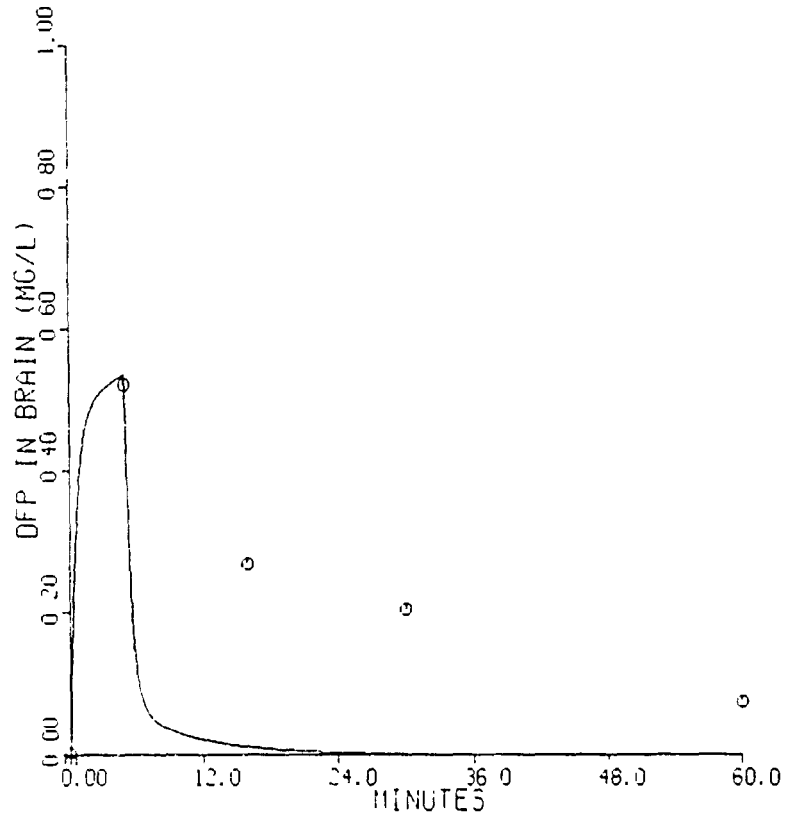


Figure 7.1-4 A & B. Time-Course of DFP Concentration in Plasma and Brain Tissue of Mice after a 5-Min Inhalation of DFP Vapor at 60 ppm.

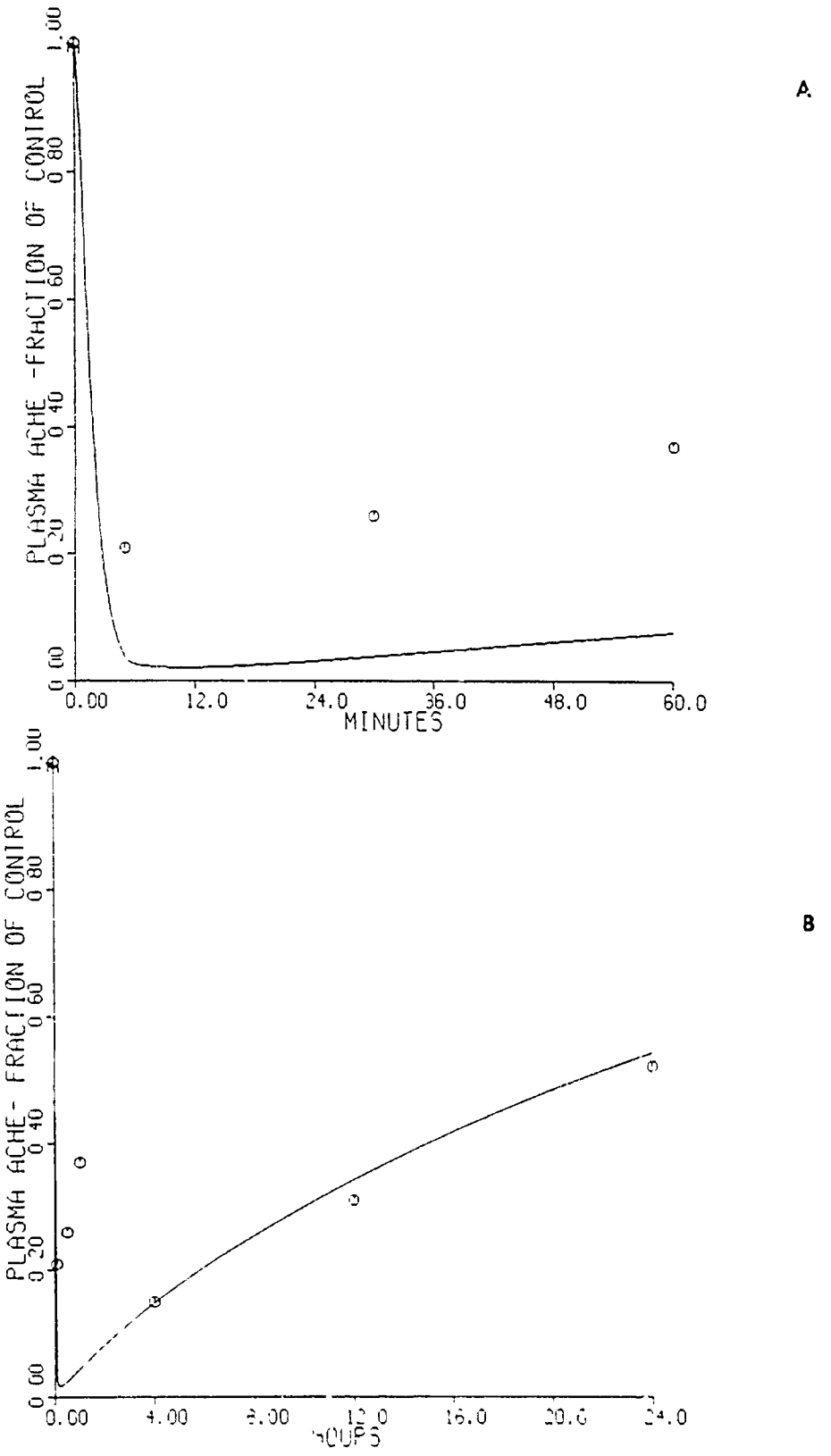


Figure 7.1-5 A & B. AChE Inhibition and Resynthesis in the Plasma of Mice after a 5-Min Inhalation Exposure of DFP Vapor at 60 ppm, Expressed as a Fraction of the Control AChE Activity.

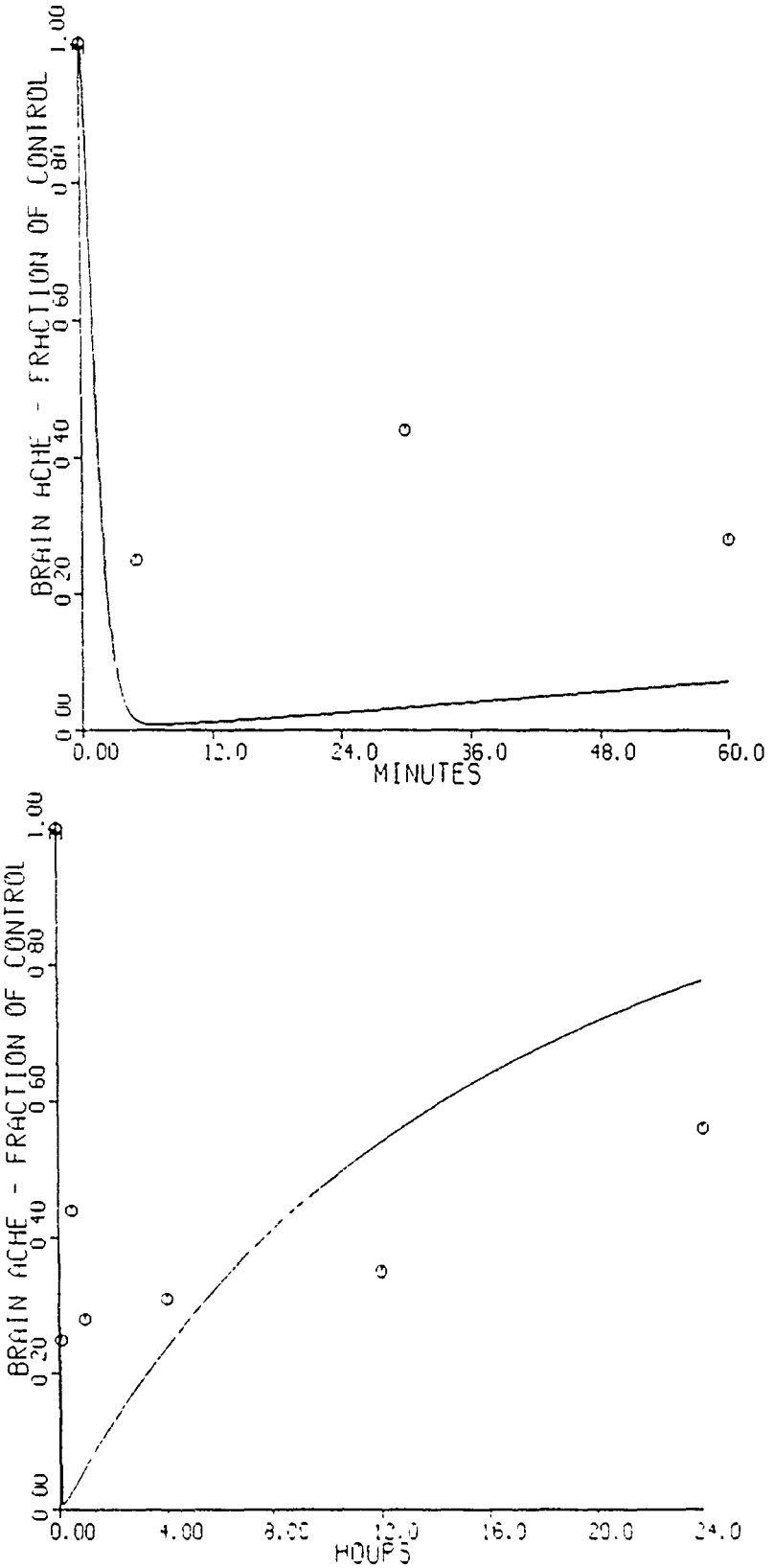


Figure 7.1-6 A & B. **AChE Inhibition and Resynthesis in Brain after a 5-Min Inhalation of DFP Vapor at 60 ppm, Expressed as a Fraction of the Control AChE Activity.**

Michalek et al. (1982) injected rats every second day with a first dose of 1.1 mg DFP/kg (in arachis oil) and subsequent doses of 0 7 mg/kg until Day 23 Five rats (four DFP and one vehicle exposed) were sacrificed 1.5 and 24 h after DFP doses 1, 2, 4, 6, 9, and 12, and at various intervals (48 and 72 h; and 7, 14, 28, and 35 days) after the last DFP administration. The brain AChE was assayed at each sacrifice point.

Traina and Serpietri (1984) used the same experimental protocol as their colleagues and determined the plasma AChE changes due to a repeated DFP dosing regime. The model predictions of DFP inhibition of plasma AChE after repeated dosing are depicted in Figure 7 1-7 for the first dose through the last dose, which included a three-day recovery following dosing. Figure 7.1-7A shows the experimental results of one sc dose of DFP at 1 1 mg/kg along with the model simulation of the inhibition and resynthesis of AChE for a 30-h time period after dosing This acute simulation is important, because the model parameters describing the first-order rate constant for DFP entering the blood compartment and the amount of new AChE synthesized are fit to the data for AChE inhibition.

The ability of the model to predict the effects of a repeated DFP dosing protocol is dictated by how well the simulation models predict the acute effects. Figure 7.1-7B shows the simulation of further DFP doses and changes in plasma AChE activity The initial 1 1 mg/kg sc dose is shown to cause a greater inhibition of AChE than the following 0.7 mg/kg doses, as would be expected. The model predictions of plasma AChE are good for the whole exposure series, except for the three-day recovery after the last dose on Day 23 In the model, AChE activity returns to 100% of control, while the experimental data overshoots the baseline AChE activity by 14% with greater experimental variability than in earlier data points The activity of AChE in plasma returned to 100% of control levels by five to six days.

The experimental values for brain AChE after repeated DFP dosing (sc) are shown in Figure 7.1-8A, B, & C, along with the model prediction for enzyme activity As was found for plasma AChE, the simulations of brain AChE inhibition and resynthesis are near the experimentally determined values The differences that occur between the predicted and expected brain enzyme activities are not in the magnitude of enzyme inhibition, but in the time at which inhibition is reached. After the dosing, the rate of AChE inhibition is controlled by the rate at which DFP enters the blood compartment and then the brain DFP absorption after sc dosing is described by a first-order rate constant If this constant is too great or too small, then there would be an over- or underprediction of AChE in both blood and brain at the 1 5- and 24-h time points. The rate at which DFP enters the blood compartment was adjusted, so that the time course of both the plasma and brain AChE inhibition data were well simulated.

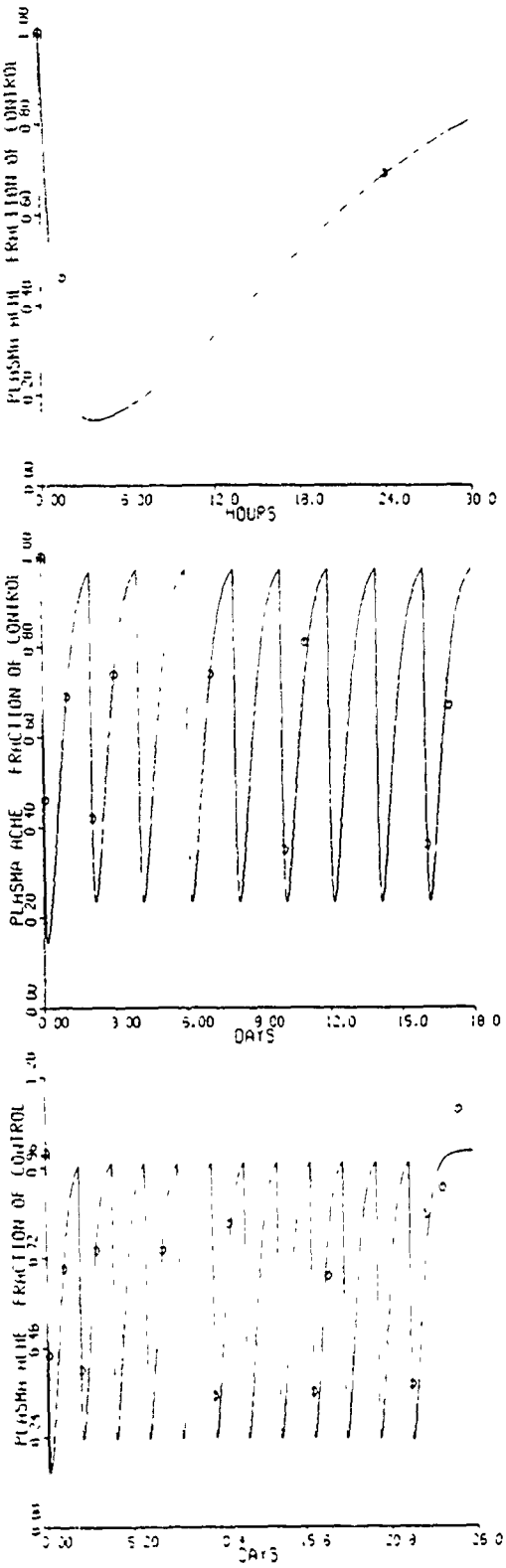


Figure 7.1-7 A, B, & C. **AChE Inhibition and Resynthesis in the Plasma of Rats Dosed Subcutaneously with an Initial Dose of 1.1 mg DFP/kg, and at 0.7 mg/kg Every Other Day Thereafter, Expressed as a Fraction of the Control AChE Activity.** Plasma AChE activity was assayed at 1.5 and 24 h after dosing with DFP.

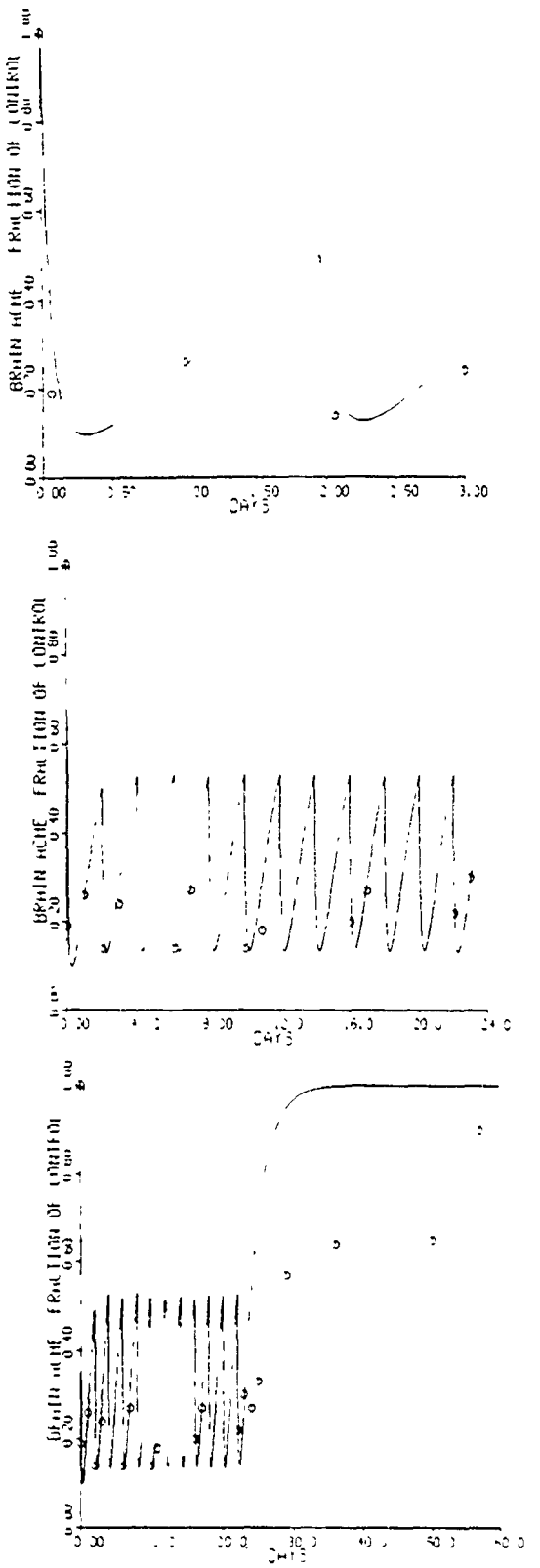


Figure 7.1-8 A, B, & C. AChE Inhibition and Resynthesis in the Brain of Rats Dosed Subcutaneously with an Initial Concentration of 1.1 mg DFP/kg, and at 0.7 mg/kg Every Other Day Thereafter, Expressed as a Fraction of the Control AChE Activity. Brain AChE activity was assayed at 1 5 and 24 h after dosing with DFP

While the model predictions for the inhibition of brain AChE during the repeated dosing scheme are good, the rate at which brain AChE returns to its pre-exposure levels is too rapid in the model. The rate of enzyme synthesis is a model fit parameter (i.e., all other parameters are held constant and the parameter-controlling enzyme synthesis is adjusted until the simulation matches the data). The experimentally determined return of brain AChE activity is much slower than the rate of enzyme resynthesis that is used in the model to predict the return of AChE activity after each dose of DFP during the repeated dosing protocol. If the rate of brain AChE synthesis were decreased to meet the rate of postexposure synthesis, then predictions of brain AChE activity after repeated DFP exposure would be underpredicted. The experimentally determined activity of AChE in the brain after each DFP dose was very reproducible, yet the levels of AChE at the 48- and 72-h time points after the last repeated DFP dose indicate there was an alteration in the mechanisms controlling enzyme synthesis.

While the model repeatedly predicted the return of brain AChE to 50% of the baseline levels by 48 h after the previous DFP dosing, the measured brain AChE activity at 48 and 72 h after the last dose of DFP was not different than the activity at 24 h. This suggests that for the OPM to predict the return of brain AChE to normal activity after a long-term repeated dosing regime, induction of resynthesis should be modeled.

DISCUSSION

A major application of PB-PK models is to accurately describe mammalian systems and to validate these models in one or more species. It then is possible to scale these models to human physiology and biochemistry, so that they can be used to improve predictions about the effects of toxicants in humans. A PB-PK simulation model has been developed to predict the acute effects in mammals of repeated exposure to the OP compound DFP. The model simulates AChE inhibition in both the plasma and brain of mice and rats after one or multiple doses of DFP.

The model will be applied next to the simulation of clinical studies where DFP was used to treat human diseases. Once the capability for simulation of AChE inhibition in humans has been demonstrated, the stage will be set for further development of the model to describe pharmacodynamic effects of chemical warfare agents.

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SECTION 8

STUDIES ON CW AGENT SIMULANTS

8.1 DETERMINATION OF THE REPEATED INHALATION TOXICITY OF CHLOROPENTAFLUOROBENZENE

E.R. Kinkead, B.T. Culpepper, L.J. Goodpaster, and H.G. Wall

INTRODUCTION

Chloropentafluorobenzene (CPFB) is a candidate material for use as a CW simulant for training purposes. Preliminary screening has indicated that CPFB provides good detectability for biological monitoring, desirable partitioning in biological tissues, acceptable physical properties, and relative biological inertness (Jepson et al., 1985).

The primary irritation hazard, sensitization potential, and acute inhalation toxicity of CPFB have been evaluated in this laboratory (Kinkead, 1987). CPFB demonstrated no potential for skin sensitization in guinea pigs; however, it did produce mild skin and conjunctival irritation in rabbits Short-term exposure to CPFB vapor posed no serious hazard by the inhalation route: All rats survived a 4-h exposure of 4.84 mg/L. It is necessary to determine the effects of repeated inhalation exposure to this material because it is anticipated that, under the conditions of intended use, individuals may be exposed to this simulant on a short-term repeated or, in the case of instructors, recurring basis.

Although the genotoxic potential of CPFB has been investigated in several studies (Tu et al., 1986; Steele, 1987), the results have been equivocal In addition, the relevancy of the high concentrations of CPFB used is difficult to assess. B6C3F1 mice were, therefore, included in this study to investigate the potential of CPFB to cause genotoxic and cytotoxic damage *in vivo*. Induction of sister chromatid exchange in bone marrow metaphase cells, induction of micronuclei in polychromatic erythrocytes, and the inhibition of bone marrow cellular proliferation were measured. The assessment of the genotoxic and cytotoxic damage in these animals was performed under subcontract by experts in cytogenetics. The needed tissue samples were collected from the animals within THRU facilities.

The rat was selected as the test species for the short-term repeated inhalation portion of this study. The species and numbers of rats per group were selected to conform with the U.S Environmental Protection Agency's Health Effects Test Guidelines (1982) and to allow for significant statistical evaluation of the results. Existing alternative methods to animal testing are inadequate for this study.

The mouse was selected as the test species for the cytogenetic studies (genotoxicity section) because the lifetime of normochromatic erythrocytes in the mouse permits analysis of the damage inducted in bone marrow over the duration of the study while the one- to two-day lifetime of polychromatic erythrocytes permits assessment of acute damage

MATERIALS AND METHODS

Animals

Upon receipt from Charles Rivers Breeding Labs (Raleigh, NC), male and female Fischer 344 (F-344) rats, 9 to 11 weeks of age, were tested and found to be in acceptable health. The animals were randomized and group housed (two to three per cage) in clear plastic cages with wood chip bedding prior to the study. The rats were housed individually and assigned to specific cage locations during the study.

The 9 to 11-week-old B6C3F1 male mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). After quality control testing, they were found to be in acceptable health. The animals were randomized and group housed (five per cage) in clear plastic cages with wood chip bedding prior to the study. During the study, rats and mice were housed in the inhalation chambers.

Water and feed (Purina Formulab #5008) were available *ad libitum* except during the inhalation exposure period and when the rats were fasted for 10 h prior to sacrifice. The light/dark cycle was set at 12-h intervals.

Test Agent

The CPFB used in this study was purchased from Aldrich Chemical Co. (Milwaukee, WI). The physical properties of CPFB are shown in Table 8.1-1.

TABLE 8.1-1. PHYSICAL PROPERTIES OF CHLOROPENTAFLUOROBENZENE

Chemical formula	C_6ClF_5
Molecular weight	202
Boiling point (°C)	117
Density (g/mL)	1.66
Vapor pressure (mmHg, 25°C)	14.1

Test Agent Quality Control

The purity of the test material was determined by capillary gas chromatography A Varian 3700 gas chromatograph equipped with an electron capture detector was used in conjunction with a Hewlett-Packard 3388 computing integrator to measure peak area and record chromatograms. CPFB was diluted in hexane to provide peak areas within the detection limits of the instrument

INHALATION TOXICITY

Generation and Analysis

CPFB vapor was generated by passing a known volume of air through a glass fritted dispersion tube immersed in a gas washing bottle containing liquid CPFB. The saturated vapor was delivered into the chamber through a stainless steel tube where it was mixed with chamber input air. Concentration was controlled by the volume of air passing through the gas washing bottle.

The chamber atmosphere was analyzed continuously using a Miran 1A (Foxboro, CT) infrared analyzer The path length varied for each chamber depending on the target concentration while the wavelength, 11.3 fm, was common to each instrument.

Exposure Regimen

Ten male and 10 female F-344 rats and six male and six female mice, age 9 to 11 weeks, were placed in each of four 690-L inhalation chambers and exposed for 6 h daily, excluding weekends (15 exposure days over a three-week period) to air only, 2.50, 0.80, and 0.25 mg CPFB/L, respectively. They were housed individually and assigned to specific exposure cage locations. The exposure cages were rotated clockwise (moving one position) within the inhalation chambers each exposure day. Test and control groups were sacrificed on the day following the 15th exposure.

Records were maintained of body weights, signs of toxicity, and mortality. At sacrifice, gross pathology was performed on all animals, and tissues (Tables 8.1-2 and 8.1-3) were harvested for histopathologic examination. Additionally, blood was drawn from the rats for hematology (Table 8.1-4) and clinical chemistry (Table 8.1-5) assays Wet tissue weights were determined on adrenals, brain, heart, kidneys, liver, lungs, ovaries (females), spleen, testes (males), and thymus from the target organ rats. From the genotoxicity mice, wet tissue weights were determined on adrenals, brain, epididymis, heart, kidneys, liver, lungs, ovaries, spleen, testes, uterus, and vagina.

TABLE 8.1-2. TISSUES HARVESTED FROM F-344 RATS FOR HISTOPATHOLOGIC EXAMINATION

Gross lesions	Mandibular lymph nodes	Uterus (females)
Thyroids/parathyroid	Mesenteric lymph nodes	Esophagus
Lungs	Eye	Stomach
Trachea	Preputial glands	Colon
Heart	Thymus	Rectum
Liver	Brain	Sternum
Spleen	Kidneys	Sciatic nerve
Duodenum	Adrenals	Skeletal muscle
Jejunum	Pancreas	Pituitary glands
Ileum	Gonads	
Urinary bladder	Nasal turbinates (3 sections)	

TABLE 8.1-3. TISSUES HARVESTED FROM B6C3F1 MICE FOR HISTOPATHOLOGIC EXAMINATION

Gross lesions	Eye	Nasal turbinates (4 sections)
Thyroid/parathyroid	Diaphragm	Uterus (females)
Lungs	Pituitary glands	Esophagus
Trachea	Larynx	Stomach
Heart	Epididymis	Colon
Liver	Salivary glands	Rectum
Spleen	Mammary gland	Sternum
Duodenum	Thymus	Sciatic Nerve
Jejunum	Brain	Skeletal muscle
Ileum	Kidneys	Preputial glands
Urinary bladder	Adrenals	Gall bladder
Mandibular lymph nodes	Pancreas	Prostate
Mesenteric lymph nodes	Gonads	Cecum
		Skin

TABLE 8.1-4. ASSAYS PERFORMED ON WHOLE BLOOD FROM F-344 RATS

Hematocrit
Hemoglobin
Red blood cell count
Total and differential leucocyte count

TABLE 8.1-5. SERUM CHEMISTRIES PERFORMED ON F-344 RATS

Creatinine	Chloride
Calcium	Phosphorus
Total protein	Alkaline phosphatase
Blood urea nitrogen	Serum glutamic-pyruvic transaminase
Serum glutamic-oxalacetic transaminase	Lactate dehydrogenase

An evaluation to determine the *in vivo* genotoxicity/cytotoxicity in the mice that were exposed in this study was performed by a subcontractor Blood was sampled serially, via tail clip, at 0, 9, 16, and 21 days of exposure for differential white blood cell counts and for micronuclei evaluation of polychromatic and normochromatic erythrocytes. Approximately 1 h after the completion of the last exposure, a 50-mg bromodeoxyuridine (BrdUrd) tablet (Boehringer Mannheim) coated partially (~70%) with paraffin (McFee et al., 1983) was implanted subcutaneously into each lightly anesthetized (Metofane®) animal The mice were killed from 18 to 19 5 h post-BrdUrd tablet implantation to ensure the presence of both first- and second-generation metaphase cells for chromosome aberration and sister chromatid exchange analysis, respectively, in a significant proportion of the animals Two hours prior to sacrifice, each animal was injected intraperitoneally with 2 mg colchicine/kg Following

CO_2 asphyxiation, the bone marrow was removed by flushing both femurs with phosphate-buffered saline (pH 7.4). The aspirated bone marrow was incubated in 0.075 M KCl for 25 min at 37°C, and fixed first with absolute methanol, then with ice-cold 3:1 methanol:glacial acetic acid. After fixation, the bone marrow was kept cold until slide preparation.

Statistical Analyses

Body weight means and associated standard errors were calculated according to the repeated multivariate analysis of variance with Scheffe pairwise comparisons (Barcikowski, 1983).

A two-factorial analysis of variance with multivariate comparisons (Barcikowski, 1983) was used to analyze the hematology, clinical chemistry, and organ weight data. The histopathology data will be analyzed using one of the following nonparametric tests: Fischer's Exact test or, if not valid, Yates' Corrected Chi-square (Zar, 1974). A probability of 0.05 inferred a significant change from controls.

RESULTS AND DISCUSSION

Chamber Analysis

The specified nominal concentrations of 0.25, 0.8, and 2.5 mg CPFB/L were sustained during the three-week exposure period. All chamber daily mean concentrations were maintained within ± 10% of the desired concentrations. Mean concentrations for each exposure chamber, along with the daily high and low mean concentrations, are provided in Table 8.1-6.

TABLE 8.1-6. ANALYSIS OF CPFB CONCENTRATIONS INHALED BY RATS AND MICE FOR 21 DAYS

Target Concentration (mg/L)	0.25 mg/L	0.80 mg/L	2.50 mg/L
Mean concentration (mg/L)			
Rats N = 16	0.25	0.79	2.52
Mice N = 15	0.25	0.77	2 55
Standard error			
Rats	0.00	0.01	0.02
Mice	0.00	0.01	0.02
Lowest daily average (mg/L)			
Rats	0.24	0.74	2.40
Mice	0.24	0.73	2 40
Highest daily average (mg/L)			
Rats	0.27	0.83	2.65
Mice	0.26	0.81	2 72

Biological Data

A total of 80 F-344 rats and 48 B6C3F1 mice were included in the three-week, 15-day inhalation toxicity study. There were no deaths resulting from the exposures. One female mouse from the 2.5 mg CPFB/L concentration group died during the BrdUrd tablet implant procedure. No signs of toxic stress were noted in any of the animals during the three-week exposure period.

Mean body weights of male and female rats exposed to 2.5 mg CPFB/L were significantly depressed during the last two weeks of the three-week study (Figure 8.1-1). Mean body weights of the male and female test mice did not differ from their respective control group (Figure 8.1-2).

Analysis of hematology parameters for male F-344 rats (Table 8.1-7) revealed no significant differences between test and control groups. Mean corpuscular volume was significantly less ($p<0.01$) than the control values for the high-concentration female rat group (Table 8.1-8), but was still within the laboratory's normal range (52.5 - 72.6 fl/red blood cells [RBC]) and very close to the value of rats in other groups. The finding was not considered to be of physiological significance because the RBC count and the hematocrit used to compute the mean corpuscular volume were within normal ranges. All other hematology values were within normal ranges.

Blood chemistry data from these rats are shown in Tables 8.1-9 and 8.1-10. Although the blood urea nitrogen values of rats exposed to 2.5 mg CPFB/L were significantly different from the controls, the 1 to 2 mg/dL difference was considered physiologically inconsequential. The total protein values of the high concentration test rats were statistically different from their respective control groups However, the differences, again, were not physiologically significant. All values were within normal ranges reported by other investigators (Ringler and Dabich, 1979).

Serum glutamic oxalacetic transaminase (SGOT) values were highest in control groups and gradually decreased as the CPFB concentration increased. The basis for this trend has not been undetermined. Since SGOT is an enzyme that increases when there is muscle damage, liver cell injury or reduced glomerular filtration, the changes in this enzyme are probably not indicative of a toxicity.

The single blood biochemistry parameter that may have pathophysiologic significance is alkaline phosphatase which was elevated in female rats in the 0.8- and 2.5-mg CPFB/L exposure groups. The elevation was statistically different from controls in the highest exposure group. Speculation that this is a functionally significant finding is based on the increased liver weights that also occurred in the two higher exposure groups of female rats. This interpretation will be reviewed and altered as appropriate when the liver histopathology has been completed. However, the preliminary interpretation is based on the anticipated findings of hepatocytomegaly which could also cause intrahepatic cholestasis and induction of alkaline phosphatase synthesis.

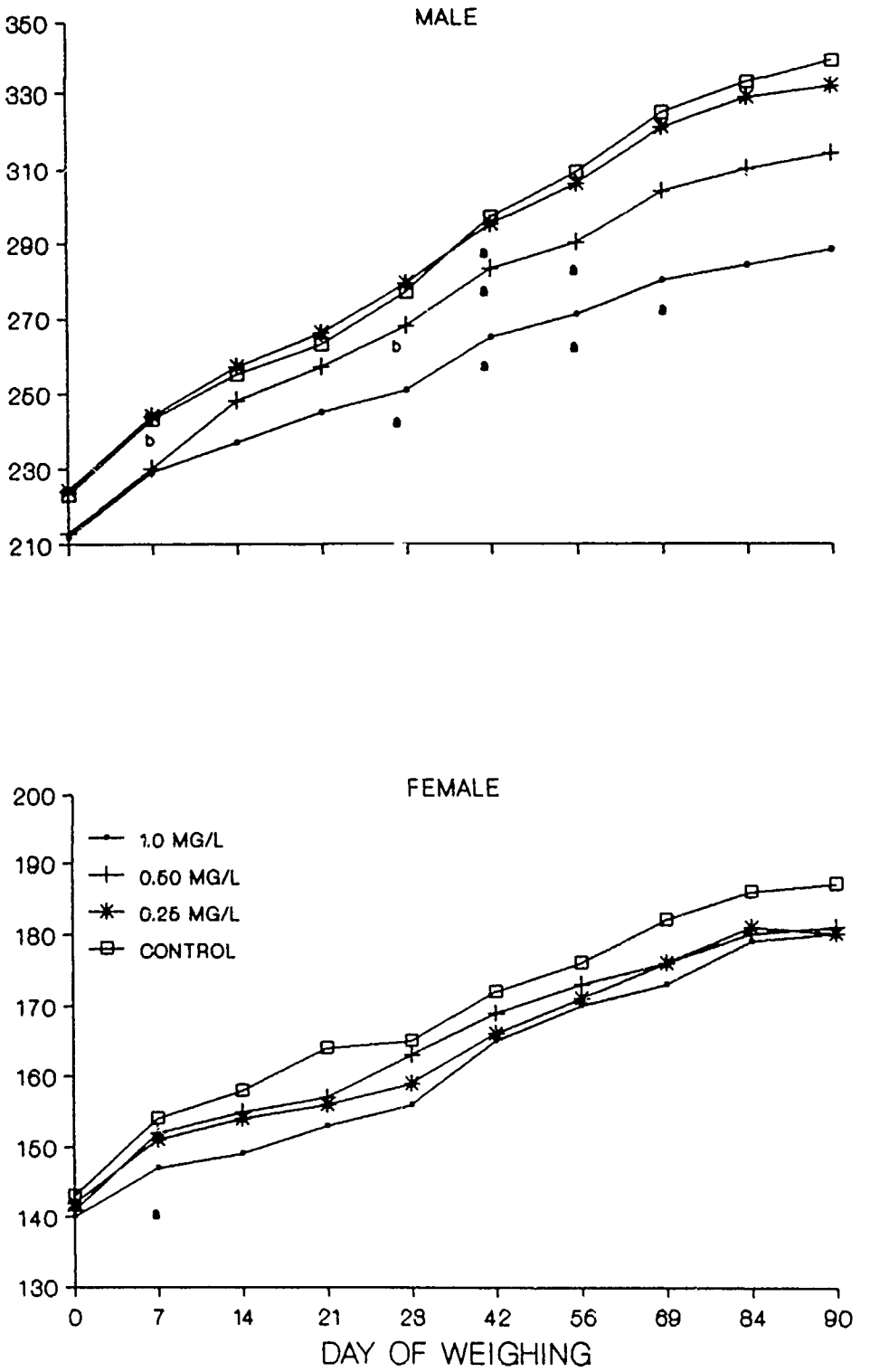

Figure 8.1-1. Body Weights of Rats Exposed to CPFB.

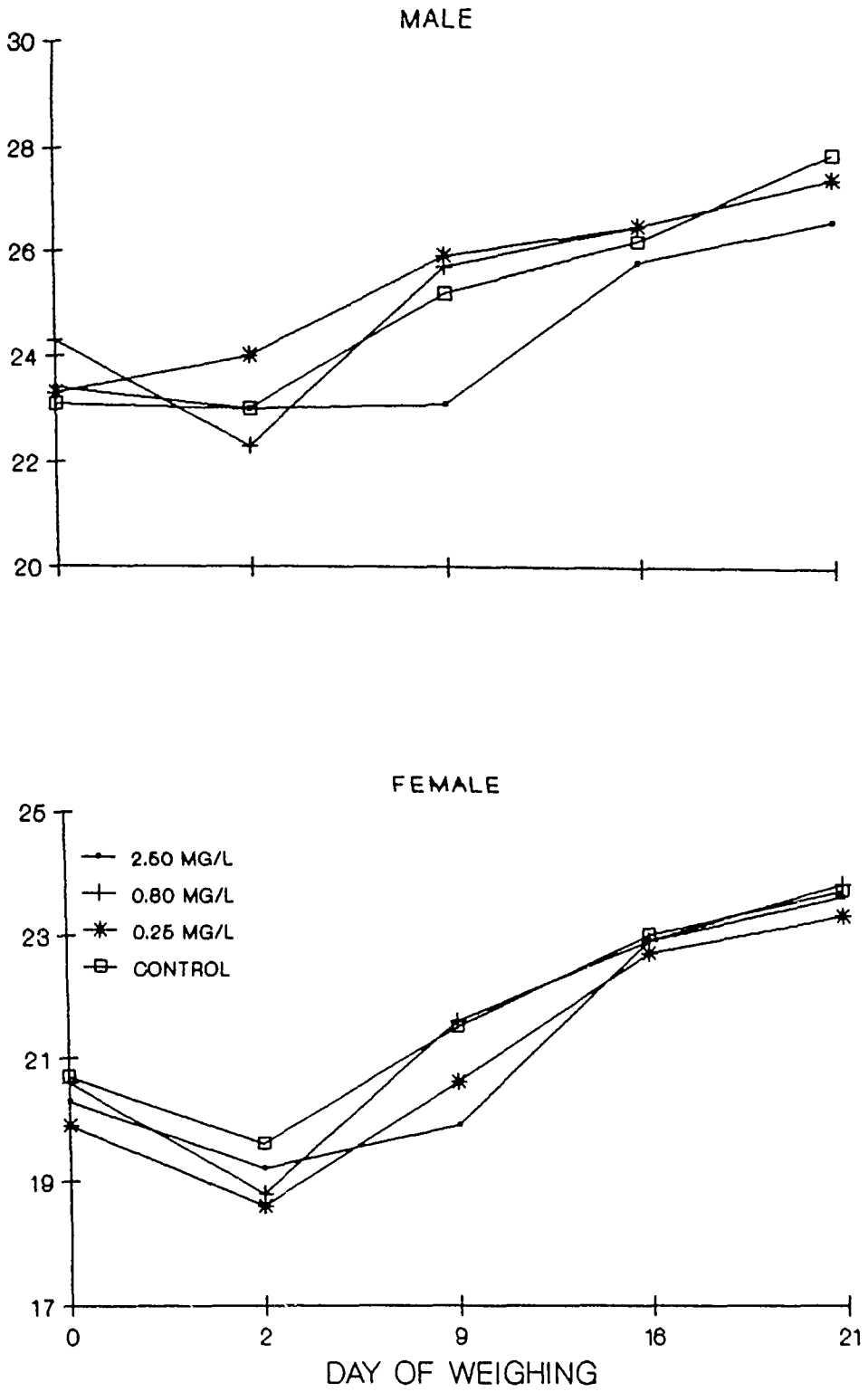

Figure 8.1-2. Body Weights of Mice Exposed to CPFB.

TABLE 8.1-7. MEAN[a] WHOLE BLOOD PARAMETERS FOR MALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L[b]	0.80 mg/L	2.50 mg/L
WBC (x 10^3 cells/mm^3)	7.50 ± 0.29	7.59 ± 0.31	8.06 ± 0.45	7.81 ± 0.28
RBC (x 10^6 cells/mm^3)	8.48 ± 0.26	8.61 ± 0.11	8.73 ± 0.10	8.59 ± 0.14
HGB (g/dL)	16.92 ± 0.24	16.96 ± 0.23	16.95 ± 0.21	16.71 ± 0.21
HCT (%)	45.33 ± 1.33	46.72 ± 0.70	46.83 ± 0.71	45.72 ± 0.83
MCV (fl)	53.40 ± 0.25	53.98 ± 0.35	53.59 ± 0.30	53.16 ± 0.25
MCH (pg)	20.07 ± 0.49	19.69 ± 0.18	19.42 ± 0.12	19.48 ± 0.14
MCHC (%)	37.52 ± 0.88	36.47 ± 0.31	36.18 ± 0.31	36.61 ± 0.30
Neutrophils (%)	15.00 ± 1.91	16.56 ± 2.58	16.90 ± 1.98	13.50 ± 2.32
Lymphocytes (%)	82.20 ± 2.02	79.00 ± 2.75	79.40 ± 2.31	83 60 ± 2 88
Monocytes (%)	3.13 ± 0 69	3 89 ± 0 79	2 89 ± 0.26	2.89 ± 0.68

[a] Mean ± S E M , N = 10.
[b] Mean ± S E M , N = 9

TABLE 8.1-8. MEAN[a] WHOLE BLOOD PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L	0.80 mg/L	2.50 mg/L
WBC (x 10^3 cells/mm^3)	6.36 ± 0.41	5.67 ± 0.32	7.01 ± 0.36	5.92 ± 0.49
RBC (x 10^6 cells/mm^3)	8.12 ± 0.06	8.13 ± 0.05	8 22 ± 0.18	8.37 ± 0.15
HGB (g/dL)	16.81 ± 0.16	16.37 ± 0.17	16.70 ± 0.24	16 61 ± 0.32
HCT (%)	49 38 ± 0.74	48 07 ± 0 51	49.26 ± 1.05	48 69 ± 0.94
MCV (fl)	60 78 ± 0.73	59 12 ± 0.37	59 84 ± 0.48	58 12 ± 0.46[b]
MCH (pg)	20.69 ± 0.17	20.14 ± 0.18	20.44 ± 0.73	19.85 ± 0 37
MCHC (%)	34.03 ± 0 25	34.05 ± 0.23	34.09 ± 1.13	34.19 ± 0.73
Neutrophils (%)	11 60 ± 1.01	13.60 ± 2.07	12.10 ± 2 05	15.10 ± 2.40
Lymphocytes (%)	85.90 ± 1.20	83.90 ± 2.50	84.60 ± 1 92	82.10 ± 2.22
Monocytes (%)	2.56 ± 0.53	2 67 ± 0 93	3 20 ± 0.70	2 70 ± 0.42

[a] Mean ± S E M , N = 10
[b] Significantly different from control at $p < 0.05$

TABLE 8.1-9. MEAN VALUES[a] OF SERUM BIOCHEMISTRIES PARAMETERS FOR MALE F-344 RATS FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L[b]	0.80 mg/L	2.50 mg/L
BUN (mg/dL)	12.9 ± 0.4	12.4 ± 0.5	12.6 ± 0.6	11.8 ± 0.3[c]
Creatinine (mg/dL)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
BUN/creatinine	46.2 ± 1.6	40.8 ± 1.9	40.6 ± 1.8	42.9 ± 2.4
Chloride (mmol/L)	100.0 ± 0.4	99.5 ± 0.4	99.6 ± 0.3	99.6 ± 0.5
Calcium (mg/L)	10.5 ± 0.1	10.6 ± 0.1	10.6 ± 0.1	10.7 ± 0.1
Phosphorus (mg/dL)	9.1 ± 0.3	9.4 ± 0.2	9.1 ± 0.2	9.5 ± 0.2
Total protein (g/dL)	5.8 ± 0.0	5.8 ± 0.1	5.9 ± 0.1	6.0 ± 0.1[d]
AST/SGOT (IU/L)	111.0 ± 3.4	107.4 ± 3.8[d]	100.2 ± 2.6[d]	97.5 ± 3.5[d]
ALT/SGPT (IU/L)	59.9 ± 1.3	60.3 ± 3.1	59.2 ± 2.3	55.7 ± 1.9
Alk. Phos. (U/L)	201.5 ± 10.6	223.9 ± 7.7	200.2 ± 7.7	205.6 ± 4.6

[a] Mean ± S.E.M., N = 10
[b] N = 9
[c] Significantly different from control at $p<0.05$
[d] Significantly different from control at $p<0.01$

TABLE 8.1-10. MEAN VALUES[a] OF SERUM BIOCHEMISTRIES PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L[b]	0.80 mg/L	2.50 mg/L
BUN (mg/dL)	13.9 ± 0.5	13.4 ± 0.5	12.7 ± 0.3	12.0 ± 0.6[b]
Creatinine (mg/dL)	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0[c]
BUN/creatinine	38.4 ± 3.1	35.8 ± 1.7	35.6 ± 1.5	40.9 ± 2.8
Chloride (mmol/L)	101.9 ± 0.4	102.0 ± 0.5	101.0 ± 0.4	101.8 ± 0.7
Calcium (mg/L)	10.4 ± 0.1	10.4 ± 0.1	10.8 ± 0.1[c]	10.7 ± 0.1
Phosphorus (mg/dL)	8.3 ± 0.3	8.0 ± 0.2	9.0 ± 0.2	8.9 ± 0.3
Total protein (g/dL)	5.7 ± 0.1	5.9 ± 0.1	5.9 ± 0.0	6.0 ± 0.1[c]
Albumin (g/dL)	3.3 ± 0.0	3.5 ± 0.1	3.5 ± 0.0	3.7 ± 0.1[c]
AST/SGOT (IU/L)	116.0 ± 3.2	101.8 ± 2.5[c]	100.9 ± 2.8[c]	107.9 ± 4.5[b]
ALT/SGPT (IU/L)	61.1 ± 1.7	57.8 ± 1.7	64.4 ± 2.4	63.3 ± 2.2
Alk. Phos. (U/L)	175.4 ± 10.5	170.3 ± 7.6	186.4 ± 5.9	213.9 ± 8.2[b]

[a] Mean ± S.E.M., N = 10
[b] Significantly different from control at $p<0.05$
[c] Significantly different from control at $p<0.01$

Concentration-related increases in relative liver weights occurred in both sexes of rats (Tables 8.1-11 and 8.1-12). Relative liver weights of the treated male rats were increased over controls by 8, 13, and 20% in the 0.25-, 0.8-, and 2 5-mg/L groups, respectively. The female rats had relative liver weight increases of 3, 8, and 18% in the respective exposure groups. Other statistical differences noted which do not appear to be treatment-related included an increase in thymus absolute and relative weights for the 0.8-mg/L exposed male rats and an increase in absolute heart weights of male rats exposed at 0.25 mg/L.

Similar concentration-related increases in relative liver weights occurred in both sexes of mice (Tables 8.1-13 and 8.1-14), with the differences being statistically significant ($p < 0.01$) at the high exposure concentration. The relative liver weights of the treated male mice were increased over controls by 16, 16, and 36% in the 0.25-, 0.8-, and 2.5-mg/L groups, respectively. The female relative liver weight increases were 12, 18, and 71% in the respective exposure groups. The heart/body weight ratios of male test mice were all lower than controls, although the difference was significant only in the group exposed at the highest concentration. This difference did not occur in the test female mice, and the significance of the weight decrease will have to be determined through histopathologic examination.

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TABLE 8.1-11. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L	0.80 mg/L	2.50 mg/L
Kidney	1.70 ± 0.03	1.77 ± 0.03	1.76 ± 0.03	1.75 ± 0.05
Ratio[b]	0 78 ± 0 01	0 79 ± 0.01	0.80 ± 0.01	0.82 ± 0.02
Heart	0.83 ± 0.02	0.91 ± 0.02[c]	0.82 ± 0.02	0.78 ± 0.01
Ratio	0.38 ± 0.01	0.41 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
Brain	1.81 ± 0.01	1.82 ± 0.02	1.80 ± 0.02	1.73 ± 0.03
Ratio	0.83 ± 0.01	0.81 ± 0.01	0.82 ± 0.01	0.81 ± 0.01
Liver	6.66 ± 0.15	7.35 ± 0.18[c]	7.57 ± 0.16[d]	7.82 ± 0.25[d]
Ratio	3.04 ± 0.04	3.27 ± 0.06[c]	3.43 ± 0.05[d]	3.66 ± 0.07[d]
Spleen	0.50 ± 0.01	0.49 ± 0.01	0.49 ± 0.01	0.46 ± 0.01
Ratio	0.23 ± 0.00	0.22 ± 0.00	0.22 ± 0.01	0.22 ± 0.00
Thymus	0.29 ± 0.01	0.31 ± 0.02	0.33 ± 0.01[c]	0.30 ± 0.01
Ratio	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.01[c]	0.14 ± 0.00
Lungs	1.52 ± 0.11	1.73 ± 0.14	1.86 ± 0.19	1.52 ± 0.12
Ratio	0.70 ± 0.06	0.77 ± 0.06	0.84 ± 0.08	0.71 ± 0.05
Adrenal	0.08 ± 0.00	0.08 ± 0.00	0 08 ± 0.00	0.08 ± 0.00
Ratio	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Testes	2.83 ± 0.04	2.90 ± 0 04	2.85 ± 0 03	2.81 ± 0.03
Ratio	1.29 ± 0.02	1 29 ± 0 02	1.29 ± 0 02	1.32 ± 0.02
Whole Body[e]	218 90 ± 2.7	224 30 ± 2 6	220 70 ± 2 6	213.00 ± 3.3

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from control at $p < 0.05$
[d] Significantly different from control at $p < 0.01$
[e] Fasted weights

TABLE 8.1-12. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L	0.80 mg/L	2.50 mg/L
Kidney	1.14 ± 0 02	1.17 ± 0 02	1.14 ± 0.02	1.17 ± 0.02
Ratio[b]	0 78 ± 0.02	0.79 ± 0 01	0.78 ± 0.01	0.81 ± 0 01
Heart	0.57 ± 0.02	0 62 ± 0.02	0.58 ± 0.01	0.56 ± 0.01
Ratio	0.39 ± 0.01	0 42 ± 0.02	0.40 ± 0.01	0.39 ± 0.01
Brain	1.68 ± 0.02	1.69 ± 0.02	1.67 ± 0.02	1.64 ± 0.02
Ratio	1.16 ± 0.01	1.14 ± 0.01	1.14 ± 0.01	1.14 ± 0.02
Liver	4.08 ± 0.07	4 28 ± 0 10	4.41 ± 0.07[c]	4.79 ± 0.11[d]
Ratio	2.80 ± 0.04	2 89 ± 0.04	3.01 ± 0.05[c]	3.31 ± 0.06[d]
Spleen	0.38 ± 0.01	0 38 ± 0.01	0.39 ± 0.02	0.36 ± 0.01
Ratio	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.25 ± 0.01
Thymus	0.27 ± 0.01	0 28 ± 0.02	0.27 ± 0.03	0.28 ± 0.01
Ratio	0.18 ± 0.01	0 19 ± 0.01	0.18 ± 0.02	0.19 ± 0.01
Lungs	1.18 ± 0.12	1.32 ± 0.09	1.26 ± 0.09	1.24 ± 0.08
Ratio	0.81 ± 0.08	0.89 ± 0.06	0.86 ± 0.06	0.86 ± 0.05
Adrenal	0.07 ± 0.00	0 08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
Ratio	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Ovary	0.12 ± 0.01	0 11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
Ratio	0.08 ± 0.01	0 08 ± 0.03	0.09 ± 0.01	0.08 ± 0.00
Whole Body[e]	145.70 ± 0.9	148 00 ± 1.7	146.30 ± 1.4	144 80 ± 1.3

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from control at $p<0.05$
[d] Significantly different from control at $p<0.01$
[e] Fasted weights

TABLE 8.1-13. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE B6C3F1 MICE FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L	0.80 mg/L	2.50 mg/L
Kidney	0.51 ± 0.02	0.53 ± 0.01	0.49 ± 0.02	0.50 ± 0.02
Ratio[b]	1.83 ± 0.05	1.92 ± 0.03	1.78 ± 0.06	1.88 ± 0.07
Heart	0 190 ± 0.01	0.160 ± 0.01	0.154 ± 0.01[c]	0.145 ± 0.01[d]
Ratio	0.688 ± 0.03	0.583 ± 0.05	0.561 ± 0.03	0.545 ± 0 02[c]
Brain	0.49 ± 0.01	0.50 ± 0.01	0.47 ± 0.01	0.51 ± 0.02
Ratio	1.78 ± 0.04	1.82 ± 0.03	1.72 ± 0.06	1.90 ± 0.07
Liver	1.42 ± 0 09	1.63 ± 0 08	1.63 ± 0.06	1.85 ± 0.08[c]
Ratio	5.13 ± 0.26	5.93 ± 0.24	5.97 ± 0.24	6.96 ± 0.32[d]
Spleen	0 09 ± 0.01	0.10 ± 0.00	0.10 ± 0 01	0.08 ± 0.00
Ratio	0.31 ± 0.02	0 36 ± 0 02	0.35 ± 0.02	0 31 ± 0.01
Lungs	0.32 ± 0.04	0.30 ± 0.02	0.30 ± 0.02	0.32 ± 0.03
Ratio	1.15 ± 0.12	1.07 ± 0.08	1.10 ± 0.07	1.21 ± 0.14
Adrenal	0.01 ± 0.00	0.01 ± 0 00	0 01 ± 0.00	0.01 ± 0.00
Ratio	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.01
Testes	0.24 ± 0.01	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.02
Ratio	0.87 ± 0.02	0.89 ± 0.04	0.80 ± 0.04	0.83 ± 0.05
Epididymis	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Ratio	0.53 ± 0.04	0 54 ± 0.05	0.63 ± 0.04	0.59 ± 0.04
Whole Body	27.66 ± 0.48	27.38 ± 0.22	27.38 ± 0 33	26 62 ± 0.46

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from control at $p<0.05$
[d] Significantly different from control at $p<0.01$

TABLE 8.1-14. ORGAN WEIGHTS[a] AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE B6C3F1 MICE FOLLOWING 21-DAY INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE

	Control	0.25 mg/L	0.80 mg/L	2.50 mg/L
Kidney	0.38 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.41 ± 0.01
Ratio[b]	1.59 ± 0.02	1.53 ± 0.04	1.46 ± 0.02	1.70 ± 0.06
Heart	0.12 ± 0 01	0.12 ± 0 00	0 13 ± 0.01	0.14 ± 0.01
Ratio	0.53 ± 0.02	0.52 ± 0.01	0.56 ± 0.03	0.56 ± 0.02
Brain	0.51 ± 0.02	0.50 ± 0.01	0.51 ± 0.00	0.45 ± 0.05
Ratio	2.14 ± 0.06	2.16 ± 0.08	2.15 ± 0.02	1.87 ± 0 21
Liver	1.10 ± 0.04	1.22 ± 0.05	1.32 ± 0.03	1.86 ± 0.08[c]
Ratio	4.67 ± 0.06	5 24 ± 0.14	5.52 ± 0.12	7.98 ± 0.25[d]
Spleen	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0 01	0.08 ± 0.01
Ratio	0.33 ± 0.01	0.36 ± 0.02	0.35 ± 0.03	0.32 ± 0.03
Lungs	0.31 ± 0.03	0.28 ± 0.02	0.23 ± 0.02	0.26 ± 0.02
Ratio	1 33 ± 0.16	1 19 ± 0 07	0 96 ± 0.08	1 10 ± 0.08
Adrenal	0 01 ± 0.00	0.02 ± 0.00	0 02 ± 0.00	0.02 ± 0.00
Ratio	0.06 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0 08 ± 0 02
Ovary	0.03 ± 0.00	0 03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
Ratio	0 11 ± 0.02	0 13 ± 0 02	0 18 ± 0.04	0.12 ± 0.02
Uterus	0.13 ± 0.01	0.15 ± 0 01	0.16 ± 0.02	0.14 ± 0.02
Ratio	0.54 ± 0.04	0.64 ± 0.04	0.65 ± 0.10	0.58 ± 0 08
Vagina	0.11 ± 0 01	0 10 ± 0 01	0 12 ± 0.01	0.10 ± 0 02
Ratio	0.44 ± 0 04	0 45 ± 0 05	0.52 ± 0 06	0.41 ± 0.08
Whole Body	23.68 ± 0 85	23 30 ± 0 56	23.83 ± 0.31	23.52 ± 0.58

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from control at p<0 05
[d] Significantly different from control at p<0 01

8.2 EVALUATION OF THE POTENTIAL OF INHALED CHLOROPENTAFLUOROBENZENE TO INDUCE SISTER CHROMATID EXCHANGES AND MICRONUCLEI FORMATION IN EXPOSED MICE

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INTRODUCTION

The purpose of this study was to evaluate in mice the *in vivo* genotoxicity/cytotoxicity of chloropentafluorobenzene (CPFB), a candidate material for use as a CW simulant for training purposes by the military. Male and female B6C3F1 mice were exposed, by inhalation, to 0 25, 0 80, and 2.50 mg/L CPFB for three weeks, excluding weekends Peripheral blood smears were prepared weekly during the exposure period and evaluated for the frequency of micronucleated erythrocytes, a genotoxic endpoint indicative of either clastogenic activity or of abnormal cytokinesis (Tice and Ivett, 1985), and of the percentage of polychromatic erythrocytes (PCE), a measure of the rate of erythropoiesis. At the completion of the exposure period, bone marrow samples were obtained and the frequency of sister chromatid exchanges (SCE), the mitotic index (MI), and the average generation time (AGT) were evaluated. SCE induction is often used as a sensitive indicator of the DNA-damaging potential of suspect agents, while the MI and the AGT can be used as a measure of the ability of the exposure to alter, in the bone marrow, either the proportion of proliferating cells and/or the rate at which they divide, respectively (reviewed in Tice and Ivett, 1985; Tice et al., 1987).

MATERIALS AND METHODS

Chemicals: Bromodeoxyuridine (BrdUrd), 50-mg tablets, was obtained from Boehringer Mannheim. Colchicine was obtained from Eli Lilly, Hoechst 33258 from American Hoechst, Giemsa from Harleco, and Metofane® from Pitman-Moore, Inc.

Groups of male and female B6C3F1 mice (six animals per group) were exposed to ambient air or to CPFB at 0.25, 0.80, and 2.50 mg/L for a three-week period (6 h per day) beginning on a Thursday and ending on a Wednesday (excluding weekends). On Day 0 and on Days 9, 16, and 21, two peripheral blood smears were prepared for micronuclei (MN) analysis by using blood obtained from the clipped tail The last sampling took place at the time of BrdUrd tablet implantation The smears were fixed in absolute methanol (approximately 5 min) Approximately 1 h after the completion of the last exposure, a 50-mg BrdUrd tablet coated partially (~70%) with paraffin (McFee et al , 1984) was implanted subcutaneously into each lightly anesthetized (Metofane®) animal In an attempt to score both chromosome aberrations (in first generation metaphase cells) and SCE (in second generation metaphase cells) in the same animal, mice were killed from 20 to 21 h post-BrdUrd tablet implantation. Approximately 2 h prior to kill time, each animal was injected intraperitoneally with

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2 mg/kg colchicine. Mice were killed by CO_2 asphyxiation and the bone marrow removed by flushing both femurs with phosphate-buffered saline (pH 7 4) The aspirated bone marrow was incubated in 0.075 M KCl for 25 min at 37°C, fixed first with absolute methanol (10 min) and then with ice-cold 3:1 methanol:glacial acetic acid After fixation, the bone marrow material was kept cold until slide preparation.

All scoring was conducted without knowledge of the exposure concentration. Each bone marrow sample was washed twice in fixative, and coded, flame-dried slides were prepared. Slides were differentially stained using a modified (Tice et al., 1978) fluorescence-plus-Giemsa technique (Goto et al., 1978). To examine the bone marrow proliferation kinetics for each mouse, 100 randomly selected metaphase cells (>20 chromosomes) were scored at ×250 magnification for replicative history (i e , the number of S phases completed since the time of BrdUrd tablet implantation) (Tice et al., 1976). Metaphase cells with intermediate differential staining patterns denoting the incorporation of BrdUrd after S phase had been initiated were scored as having completed that S phase. Twenty-five second generation metaphase cells per animal (four mice per exposure group) were scored at ×1000 magnification for SCE. with selection being based on good chromatid differential staining, a lack of overlapping chromosomes, and chromosome number (40 ± 3 chromosomes).

For MN evaluation, coded peripheral blood smears were stained with the DNA-specific stain, acridine orange (AO), as described in Tice et al. (1987). Each slide was scanned at ×800 to ×1000 magnification using epi-illuminated fluorescence microscopy (450 to 490 nm excitation, 520 nm emission) for the number of MN PCE among 1000 PCE, the number of MN normochromatic erythrocytes (NCE) among 1000 NCE, and the percentage of PCE in 1000 erythrocytes. At the same time, information on the number of erythrocytes with 0, 1, 2, etc , MN was retained.

Positive Controls: To evaluate scorer reliability, coded slides obtained from male B6C3F1 mice exposed to 7,12-dimethylbenz(a)anthracene (DMBA) (2.5 mg/kg, delivered in corn oil via intraperitoneal injection) 42 h prior to bone marrow sampling were included among the CPFB bone marrow slides. In a similar fashion, coded peripheral blood smears from male C57B1/6 mice exposed to benzene (100 ppm, 6 h/day for 20 weeks, five days per week) were included among the peripheral blood smears obtained from CPFB-exposed mice.

Statistical Analysis: For all statistical analyses, the level of significance was established at an alpha of 0.05. The data were first analyzed using a Bloon-Forsythe two-way ANOVA to determine whether a significant sex difference in response occurred If not significant, the subsequent analysis was based on pooled data; if significant, each sex was evaluated separately. A one-tailed trend test (Margolin et al , 1986) was used to determine if a treatment-related increase occurred for SCE or MN erythrocyte data. For AGT, MI, and %PCE data, a two-tailed trend test was used to determine if a treatment-related effect occurred. For SCE, AGT, MI, and %PCE data, the ability of the treatment to

affect the group mean values was analyzed by the trend test using individual animal responses. For MN data, the number of MN-PCE or MN-NCE was summed across animals within each exposure group and analyzed by a one-tailed trend test (Margolin and Risko, 1986). For statistically significant responses, pairwise comparisons between each exposure group and the corresponding concurrent control group were conducted using the t test or Pearsons Chi square test (with the alpha level Bonferroni-corrected for multiple comparisons), as appropriate, to determine the minimal effective dose for each endpoint. For SCE data, an additional statistical test, the Dispersion test (Margolin and Shelby, 1985), based on the ratio of the sample variance to the sample mean, was used to evaluate the effect of the exposure to CPFB on the intercellular distribution of SCE within each animal. This statistical approach is very sensitive to the presence of outlier cells (i e., cells with a significantly elevated number of SCE within a population of cells having an otherwise control SCE rate). These cells can be induced if either the genotoxic activity of the agent is cell stage specific or if the distribution of the agent is nonuniform within the bone marrow. For the analysis of cellular proliferation kinetics, the proportion of first, second, and third generation metaphase cells in each bone marrow sample was transformed into an AGT (Ivett and Tice, 1982), where AGT is equal to BrdUrd exposure duration/replicative index (RI). The RI is equal to one time the frequency of first generation metaphase cells plus two times the frequency of second generation metaphase cells plus three times the frequency of third generation metaphase cells plus . . . (Schneider and Lewis, 1981). In addition to the trend test for evaluating the number of micronucleated cells, the proportions of cells with 0, 1, 2, etc., MN were evaluated to determine whether the intercellular distribution of events was significantly affected by the exposure to CPFB.

RESULTS

The SCE/cell, the H value, the AGT, and the MI data from male and female B6C3F1 mice exposed to CPFB are presented in Table 8.2-1. A two-way analysis of the data in male and female mice revealed a significant difference in SCE ($P = 0.0693$) response between the sexes. A significant sex by dose interaction term was observed for AGT data ($P = 0.0417$), thereby requiring the separate statistical analysis of male and female data. Exposure to CPFB at concentrations of 0.25, 0.80, and 2.50 mg/L for three weeks (6 h per day, excluding weekends) did not induce a significant increase in the frequency of SCE in the bone marrow of male and female mice ($P = 0.0688$). Similarly, a dispersion analysis of the SCE data also indicated a lack of damage induced by CPFB. In the bone marrow, the rate of cellular proliferation was not altered in either male ($P = 0.1138$) or female ($P = 0.0911$) mice, but the number of proliferating cells was significantly increased ($P = 0.0010$). As expected, a significant increase in SCE frequency was detected in the bone marrow of positive control mice injected with DMBA ($P < 0.0001$).

TABLE 8.2-1. GROUP BONE MARROW CYTOGENETIC DATA FOR B6C3F1 MICE EXPOSED TO CPFB

Concent. (mg/l)	Sex	SCE[a] Mean	±	S.E.M.	N	H[b] Stat. Mean	±	S.E.M.	N	AGT[c] (h) Mean	±	S.E.M.	N	MI[d] (%) Mean	±	S.E.M.	
0	M	5.27	±	1.079	4	1.18	±	0.110	4	14.24	±	0.935	6	2.70	±	0.425	6
	F	7.26	±	1.040	4	1.80	±	0.556	4	14.75	±	0.609	6	2.68	±	0.311	6
	M + F	6.26	±	0.789	8	1.49	±	0.287	8					2.69	±	0.251	12
0.25	M	4.11	±	0.111	4	0.85	±	0.129	4	14.72	±	1.374	6	2.72	±	0.334	6
	F	5.87	±	0.804	4	1.53	±	0.253	4	16.74	±	0.804	6	1 87	±	0 431	6
	M + F	4.99	±	0.502	8	1.19	±	0.184	8					2.29	±	0.290	12
0.80	M	5.95	±	0.454	4	1.31	±	0.183	4	14.64	±	0.920	6	2.43	±	0.612	6
	F	5.79	±	0.440	4	1.48	±	0.381	4	16.64	±	0.750	6	3.10	±	0.509	6
	M + F	5.87	±	0.294	8	1.39	±	0.198	8					2.77	±	0.393	12
2.50	M	4.69	±	0.143	4	1.44	±	0.136	4	16.48	±	0.968	6	4.13	±	0.313	6
	F	4.88	±	0.516	4	1.07	±	0.056	4	13.68	±	0 772	5	3.58	±	0.477	5
	M + F	4.78	±	0.250	8	1.26	±	0.098	8					3.82	±	0.258[e]	11
DMBA positive control data (mg/kg)																	
0	M	5.25	±	0.310	4	0.93	±	0.177	4	12.84	±	0.144	4	2.75	±	0 343	4
2.5	M	16.40	±	1.315[f]	4	3.13	±	1.180[f]	4	13.18	±	0.211	4	3.95	±	0.378	4

[a]SCE = Sister chromatid exchange/cell frequency based on 25 cells per mouse
[b]H = SCE variance/SCE mean
[c]AGT = Average generation time in hours based on 100 metaphase cells per mouse
AGT = BrdUrd exposure time/1 x frequency of M1 + 2 x frequency of M2 + 3 x freqency of M3 metaphase cells
[d]MI = Mitotic Index The MI is based on scoring 1000 nucleated cells per mouse
[e]Significantly different at alpha = 0 05 using a one-tailed Student's t test, based on separate group variances.
[f]Significantly different at alpha = 0 05 using a one-tailed trend t test, based on individual mouse data

Data collected from the scoring of the peripheral blood smears are presented in Table 8.2-2. Because of a significant sex difference in MN-NCE numbers at Day 0 ($P = 0.0174$) and in MN-PCE numbers at Day 9 ($P = 0.0020$) and at Day 21 ($P = 0.0002$), MN data were analyzed without pooling between the sexes. For male mice, at every sample time, exposure to CPFB failed to induce an increase in the number of MN-PCE ($P = 0.08$) or in the number of MN-NCE ($P > 0.11$). For female mice, significant MN-NCE frequencies were not significantly elevated at any sample time ($P = 0.24$), while the number of MN-PCE was significantly increased at Day 16 only ($P = 0.0038$). Since none of the micronucleated erythrocytes in any of the control or CPFB-treated mice contained more than one MN, an evaluation of the distribution of MN among cells was not conducted. Significant increase in MN-PCE and MN-NCE populations was detected in the bone marrow of mice used as a positive control ($P < 0.0001$). After pooling %PCE data between male and female mice ($P > 0.12$), a trend test analysis of these data indicated a significant depression in the rate of erythropoiesis at Days 9 ($P = 0.0258$) and 21 ($P = 0.0078$)

DISCUSSION

The endpoints selected for evaluation in this study cover a range of cytotoxic and genotoxic biologic responses – from indicators for DNA damage, both base and strand breakage (SCE and MN, respectively), to abnormal cytokinesis (MN), to effects on cell proliferation (AGT and MI), to alteration in the rate of erythropoiesis (%PCE) In the bone marrow, the AGT measures the average cell cycle duration for the proliferating cell populations while the MI assessment is a measure of the proportion of proliferating cells. The percentage of PCE in peripheral blood is a measure of the overall rate of erythropoiesis. Genotoxic damage can and does lead to cellular toxicity, measured either as a loss in reproductive capacity (Joshi et al., 1982) or as a delay in cell growth (Painter and Howard, 1982). However, significant suppression of bone marrow cellular proliferation can occur in the absence of any measurable genotoxicity (Tice et al., 1987), indicating that nongenotoxic mechanisms also can be involved. Bone marrow was selected as the target organ in this study for several reasons. First, bone marrow is the major site of hematopoiesis and is comprised of several functionally distinct cell populations. Bone marrow damage can occur across cell lineages with all proliferating cell populations affected, or the damage can be relatively lineage- and cell-type specific. Thus, effects on stem cells, proliferating cells, or differentiating cells can be assessed concurrently. Second, the high rate of cell turnover makes the bone marrow a sensitive target for carcinogenic/mutagenic chemicals. Third, experimentally obtained data indicate the relevance of bone marrow damage to future adverse health effects. Cytotoxic damage to bone marrow cells can be related easily to diseases such as pancytopenia or anemia while genotoxic damage can be correlated with tumor induction

TABLE 8.2-2. GROUP PERIPHERAL BLOOD MICRONUCLEUS DATA FOR B6C3F1 MICE EXPOSED TO CPFB

MN-PCE[a]

Exposure Duration (days)	Sex	Dose = 0			Dose = 0.25			Dose = 0.80			Dose = 2.50			Positive Control		
		Mean	S.E.M.	N[b]	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	
0	M	2.33	± 0.667	6	2.00	± 0.516	6	3.33	± 0.843	6	2.67	± 0.955	6	28.00	± 4.412	6[c]
	F	1.17	± 0.307	6	2.67	± 0.760	6	2.67	± 0.494	6	2.33	± 0.422	6			
9	M	4.33	± 0.333	6	2.00	± 0.577	6	2.83	± 0.477	6	2.17	± 0.401	6	25.33	± 3.528	6[c]
	F	1.17	± 0.601	6	2.67	± 0.803	6	1.17	± 0.477	6	1 33	± 0 333	6			
16	M	3.17	± 0.601	6	3.33	± 0.174	6	3.33	± 1.116	6	2.50	± 0.563	6	27 50	± 3.845	6[c]
	F	2.00	± 0.683	6	1.83	± 0.543	6	2.00	± 0.516	6	4.00	± 0.516	6[d]			
21	M	3.00	± 0.365	6	3.67	± 0.494	6	2.00	± 0.258	6	2.83	± 0 477	6	28 00	± 2 745	6[c]
	F	1.50	± 0.428	6	1.67	± 0.422	6	1.50	± 0.563	6	1.80	± 0.342	5			

[a]MN-PCE = Micronucleated polychromatic erythrocytes

[b]Group mean values are of micronucleated erythrocytes per 1000 erythrocytes ± the standard error of the mean among N animals

[c]Significantly different from concurrent control data at α = 0 05 based on a one-tailed Pearson chi-square test, based on pooled mouse data

[d]Significantly different from concurrent control data at α = 0 05 based on a one-tailed trend test, based on pooled MN data

TABLE 8.2-2. CONTINUED

MN-NCE[a]

Exposure Duration (days)	Sex	Dose = 0 Mean	S.E.M.	N[b]	Dose = 0.25 Mean	S.E.M.	N	Dose = 0.80 Mean	S.E.M.	N	Dose = 2.50 Mean	S.E.M.	N	Positive Control Mean	S.E.M.	
0	M	2.83 ±	0.601	6	2.17 ±	0.477	6	2.50 ±	0671	6	1.67 ±	0.333	6	8.33 ±	1.022	6[c]
	F	1.67 ±	0.615	6	1.83 ±	0.477	6	1.17 ±	0.543	6	0.83 ±	0.307	6			
9	M	2 00 ±	0.516	6	1.50 ±	0.428	6	1.50 ±	0.563	6	0.83 ±	0.477	6	9.33 ±	1.382	6[c]
	F	1.83 ±	0.477	6	2.33 ±	0.494	6	2.17 ±	0.980	6	1.17 ±	0.167	6			
16	M	2.67 ±	0.919	6	1.17 ±	0.307	6	2.17 ±	0.477	6	2.33 ±	0.760	6	11.33 ±	1.022	6[c]
	F	2.50 ±	0.719	6	1.67 ±	0.333	6	2.00 ±	0.516	6	1.67 ±	0.422	6			
21	M	1.17 ±	0.401	6	1.33 ±	0.422	6	1.83 ±	0.654	6	1.67 ±	0.333	6	10 33 ±	1.856	6[c]
	F	1.83 ±	0.703	6	1.67 ±	0.558	6	2.17 ±	0.654	6	1.60 ±	0.228	5			

[a]MN-PCE = Micronucleated polychromatic erythrocytes

[b]Group mean values are of micronucleated erythrocytes per 1000 erythrocytes ± the standard error of the mean among N animals

[c]Significantly different from concurrent control data at α = 0 05 based on a one-tailed Pearson chi-square test, based on pooled mouse data

[d]Significantly different from concurrent control data at α = 0 05 based on a one-tailed trend test, based on pooled MN data

TABLE 8.2-2. CONTINUED

%PCE[a]

Exposure Duration (days)	Sex	Dose = 0 Mean	S.E.M.	N[b]	Dose = 0.25 Mean	S.E.M.	N	Dose = 0.80 Mean	S.E.M.	N	Dose = 2.50 Mean	S.E.M.	N	Positive Control Mean	S.E.M.	
0	M	2.63	± 0.762	6	2.53	± 0.355	6	2.88	± 0.494	6	2.25	± 0.402	6	2.12	± 0.381	6[c]
	F	2.60	± 0.392	6	2.07	± 0.409	6	3.28	± 0.356	6	2.62	± 0.335	6			
	M + F	2 62	± 0.409	12	2.30	± 0.267	12	3.08	± 0.296	12	2.43	± 0.256	12			
9	M	2.18	± 0.180	6	2.13	± 0.178	6	2.32	± 0.375	6	1.82	± 0.202	6	2.07	± 0.397	6[c]
	F	2.63	± 0.216	6	2.58	± 0.257	6	2.25	± 0.226	6	2.03	± 0.199	6			
	M + F	2.41	± 0 150	12	2.36	± 0.164	12	2.28	± 0.209	12	1.92	± 0.139	12[d]			
16	M	3.53	± 0 312	6	2 77	± 0.256	6	5.30	± 2.602	6	2 20	± 0.167	6	2 02	± 0 196	6[c]
	F	2.10	± 0.173	6	3.35	± 0.385	6	2.05	± 0.295	6	2.27	± 0.167	6			
	M + F	2.82	± 0.375	12	3.06	± 0.238	12	3.68	± 1.341	12	2.23	± 0.113	12			
21	M	2 03	± 0 194	6	2.70	± 0.235	6	1.82	± 0.180	6	1.75	± 0.184	6	2.32	± 0.280	6[c]
	F	1.62	± 0.168	6	2.50	± 0.257	6	2.03	± 0.291	6	1.20	± 0.198	5			
	M + F	1 82	± 0.138	12	2.60	± 0.169	12	1.92	± 0.166	12	1.50	± 0.159	11[d]			

[a]%PCE = Percentage of polychromatic erythrocytes

[b]Group mean values are of micronucleated erythrocytes per 1000 erythrocytes ± the standard error of the mean among N animals

[c]Significantly different from concurrent control data at α = 0 05 based on a one-tailed Pearson chi-square test, based on pooled mouse data

[d]Significantly different from concurrent control data at α = 0 05 based on a one-tailed trend test, based on individual mouse data

Exposure of male and female B6C3F1 mice to CPFB, 6 h per day, for three weeks (excluding weekends) failed to induce an increase in SCE (based on the mean or on the intercellular dispersion)

However, in female but not male mice, a slight but statistically significant increase in MN-PCE frequency was detected at a single sample time (Day 16). Although a repeat study is required to verify the accuracy of this finding, the weakness of the response combined with the lack of supporting data at Day 9 and/or Day 16 suggests that the response is spurious. The lack of a significant increase in SCE and the nature of the MN response indicated that an evaluation of chromosomal aberration frequencies in bone marrow cells is unwarranted. Exposure to CPFB did not result in an altered rate of cellular proliferation in bone marrow but it did depress the rate of erythropoiesis at Days 9 and 21 and it did increase the MI in mice sampled at the end of the exposure period These altered responses indicated that exposure to CPFB resulted in signs of systemic stress in mice. The absence of significant and internally reproducible genotoxic damage in bone marrow of mice suggests that CPFB is not genotoxic.

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SECTION 9

SUBCHRONIC TOXICITY STUDIES OF NAVY MATERIALS

9.1 DETERMINATION OF THE SUBCHRONIC TOXICITY OF CYCLOTRIPHOSPHAZENE HYDRAULIC FLUID BY 21-DAY REPEATED DERMAL EXPOSURE

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INTRODUCTION

The Navy has developed candidate hydraulic fluids with chemical structures of cyclotriphosphazene (CTP) cyclic esters. Acute toxicity studies demonstrated that this hydraulic fluid is nontoxic by oral or dermal administration (unreported data). Eye and skin irritation tests, as well as skin sensitization tests, also proved negative (Kinkead and Bowers, 1985). The hydraulic fluid was not detected in the blood or urine of rats following exposure by aerosol inhalation or dermal contact (unreported data). The oral LD_{50} of tolyltriazole, a corrosion inhibitor additive to this hydraulic fluid, in rats is 675 mg/kg (Huntingdon Research Center Report, 1972).

This report addresses the effects of repeated exposures by the dermal route. These experiments were designed to measure the toxic effects associated with repeated or continuous exposure to CTP over a limited time. An additional objective was to determine a "no observed effect" treatment dosage of the compound. These studies were not designed to identify those effects that have a long latency period (e.g , carcinogenicity, decreased life expectancy). The selected species and group sizes used in these studies conform to the U S. Environmental Protection Agency's Health Effects Test Guidelines (1982).

MATERIALS AND METHODS

Test Agent

The CTP cyclic ester hydraulic fluid contained 0.1% tolyltriazole. It was supplied by the Toxicology Detachment of the Naval Medical Research Institute (NMRI/TD), Wright-Patterson Air Force Base, OH The pertinent data on these materials are provided below

CTP ester:

NMRI/TD No.	4341-1
Vapor Pressure, mmHg	
65°C	0 49
149°C	12 0
Specific Gravity (g/mL)	1 445

Tolyltriazole:

Chemical Formula	$C_7H_7N_3$
CAS No.	29385-43-1
Synonyms	Methylbenzotriazole
	1,2,3-Triazole
	(methylphenyl)

This hydraulic fluid is a mixture of parent compound isomers including dimers, trimers, and tetramers of CTP. The approximate molecular weight of the fluid is 1000 g/mole.

Test Agent Quality Control

A Varian 3700 gas chromatograph equipped with a flame ionization detector and a 50-m, 5% phenylmethyl silicone capillary column was utilized in conjunction with a Hewlett-Packard 3388 computing integrator to measure peak area and record chromatograms of the test material. Profiles were obtained of the material as received, as aerosolized, and as residue from the nebulizer system.

Animals

Male and female New Zealand White rabbits, 2 to 3 kg in weight, were obtained from Clerco Research Farms (Cincinnati, OH) for use in the dermal studies. Quality control evaluations confirmed the satisfactory health of the proposed study animals. The rabbits were housed individually in wire-bottom, stainless-steel cages. Water and food (Purina Rabbit Chow #5320 and/or MannaPro Rabbit Family Ration) were available *ad libitum*. Rabbits were maintained on a 12-h light/dark cycle.

METHODS AND EXPERIMENTAL EVALUATIONS

A detailed description of the methods and experimental evaluations performed for this dermal toxicity study has been published (Kinkead et al., 1988).

RESULTS

A total of 80 New Zealand White rabbits were used in the three-week dermal toxicity study. Two rabbits were euthanatized during the course of the study, one rabbit treated with 1.00 g/kg and a second treated with 0 25 g/kg, following accidental injury during the daily handling procedures. No behavioral abnormalities or signs of toxic stress were observed in the study animals at any time during the three-week treatment regimen. All groups gained weight during the course of the study (Table 9 1-1) Statistical analysis of body weights confirmed that there were no treatment-related differences.

Analysis of hematology data (Tables 9.1-2 and 9 1-3) revealed no apparent treatment-related effects, and all group means for these parameters were within the normal ranges for the age and

TABLE 9.1-1. MEAN BODY WEIGHTS[a] (kg) OF NZW RABBITS DURING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE HYDRAULIC FLUID

Sex	Dose Group	Body Weights (kg)			
		Day 0	Day 7	Day 14	Day 21[b]
Male	Control	2.7 ± 0.05	2.8 ± 0 06	2.9 ± 0.08	3.0 ± 0.07
	0.25 g/kg	2.8 ± 0.05	2.9 ± 0.06	3 0 ± 0.08	3.1 ± 0.09[c]
	0.50 g/kg	2.7 ± 0 06	2.8 ± 0 06	2.9 ± 0.07	3.0 ± 0.08
	1.00 g/kg	2.8 ± 0 06	2.9 ± 0 07[c]	3.0 ± 0.08[c]	3.0 ± 0.08[c]
Female	Control	2.7 ± 0.03	2.8 ± 0.05	2.9 ± 0 05	3.0 ± 0 06
	0.25 g/kg	2.9 ± 0.04	2.9 ± 0.06	3 1 ± 0.07	3.3 ± 0.07
	0.50 g/kg	2.8 ± 0.05	2.9 ± 0.05	3.0 ± 0.07	3.1 ± 0.06
	1.00 g/kg	2.8 ± 0.06	2.8 ± 0 06	2 8 ± 0.06	3 0 ± 0 09

[a] Mean ± S E M , N = 10 except where noted
[b] Fasted weights
[c] N = 9

species of these animals (Wolford et al , 1986) Clinical chemistry data (Tables 9 1-4 and 9.1-5) indicated no significant differences from controls for any of the parameters examined

Organ weights, measured at necropsy (Tables 9 1-6 and 9 1-7), identified the kidneys, liver and thymus of the test rabbits as different from those organs in the respective control group. The absolute kidney weights and the kidney to body weight ratios of all test groups of both sexes were higher than their respective controls; however, the increases were statistically significant only for the 0.25 (absolute weight and ratio, both sexes) and 0 5 (ratio only) g CTP/kg male treatment groups. Similarly, with one exception, the absolute liver weights and the liver to body weight ratios of test groups of both sexes were higher than their respective controls; statistical significance was limited to the 0.25 and 0.5 g CTP/kg treatment groups. The high dose group of male rabbits exhibited a mean thymus weight and mean thymus to body weight ratio significantly lower than the male control group ($p<0.01$); however, there were high coefficients of variation for these means.

The gross examinations and correlated histopathologic examinations disclosed one low dose CTP-exposed male rabbit to have a vertebral fracture, one median dose CTP-exposed rabbit to have pampiniform plexus congestion, and one median dose CTP-exposed female rabbit to have liver necrosis. Subacute typhlitis, involving from 56 to 100% of each dose/sex group, and high incidences of cecal pinworms (*Passalurus ambiguus*), mesenteric and ileal lymphoid hyperplasia, and subacute ileitis were detected Scattered cases (four total) of pulmonary abscessation occurred The abscesses were consistent with those associated with Pasteurellosis Pulmonary edema was diagnosed in two or fewer male rabbits at each of the three CTP treatment groups, and in three female controls, two females from the median CTP dose group, and one female in the high CTP dose group Fifty percent of the female rabbits in the control, median dose CTP, and high dose CTP groups had pulmonary congestion. The incidence of pulmonary congestion was 20% or less in all other rabbit dose and sex groups Renal tubular mineralization occurred in one male rabbit from each dose group, including

controls, and in one, five, four, and three female rabbits from the controls, low, median and high CTP dose groups, respectively. Statistical analyses indicated that female rabbits had higher incidences of ileal and cecal subacute inflammation ($p<.01$), dilated renal tubules ($p<.05$), renal tubular mineralization ($p<.05$), mandibular lymph node lymphoid hyperplasia ($p<.01$), and pulmonary congestion ($p<.01$). However, increases in incidences of histologic lesions were not dose-related.

DISCUSSION

Repeated dermal applications of CTP to male and female rabbits for 21 days (15 applications) resulted in no observable signs of toxic stress. No treatment-related effects were noted in body weights measured during the study nor in blood parameters examined at the conclusion of the treatment period. Although the absolute liver weights in some treatment groups were statistically different from controls, the difference was negated when compared by mean body weight. Whenever organ weight/body weight was significantly different from control, the effect was found by statistical analysis to be unrelated to treatment level. Histopathologic examination of tissues from both sexes of rabbits following termination of treatment did not disclose any lesion that could be attributed to CTP exposure. Congestion and edema in the respiratory tract were considered to be agonal or postmortem changes. Other lesions were considered to be due to bacterial infection (pulmonary abscesses), parasitic infection: typhlitis, ileitis, colitis, lymphadenitis, mesenteric, and ileal hyperplasia; or to represent mild background lesions.

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TABLE 9.1-2. MEAN[a] WHOLE BLOOD PARAMETERS FOR NZW MALE RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
WBC (x 10^3 cells/mm^3)				
Pre-exposure	6.26 ± 0.64	6.48 ± 0.37	5.54 ± 0 35	6.14 ± 0.50
Postexposure	7.38 ± 0.99	6.30 ± 0.17	5.72 ± 0.43	6.26 ± 0.74
RBC (x 10^6 cells/mm^3)				
Pre-exposure	5.95 ± 0.15	5.53 ± 0.16	5.73 ± 0.17	5 62 ± 0.08
Postexposure	6.11 ± 0.07	5.77 ± 0 16	5.88 ± 0.17	5.84 ± 0.09
HGB (g/dL)				
Pre-exposure	12.88 ± 0.24	12.14 ± 0.24	12.50 ± 0.29	12.30 ± 0.16
Postexposure	13.44 ± 0.23	13.20 ± 0.13	13.04 ± 0.40	12.80 ± 0.09
HCT (%)				
Pre-exposure	37.52 ± 0.81	35.46 ± 0.88	36.16 ± 0.84	35.90 ± 0 47
Postexposure	38.60 ± 0.68	37.92 ± 0.33	37.46 ± 1.21	37.16 ± 0.36
MCV (mm^3)				
Pre-exposure	63.02 ± 0.53	64.14 ± 0.91	63.20 ± 1.05	64.10 ± 0.74
Postexposure	63.04 ± 0.91	64.34 ± 0.91	63.68 ± 0.99	63.60 ± 0.59
MCH (10^{-12}/cells/mm^3)				
Pre-exposure	21.62 ± 0.29	21.96 ± 0.29	21.82 ± 0.29	21.86 ± 0.21
Postexposure	21.96 ± 0.29	22.46 ± 0.35	22.20 ± 0 30	21.94 ± 0.25
MCHC (%)				
Pre-exposure	34.28 ± 0.24	34.26 ± 0.28	34.56 ± 0 26	34.26 ± 0.23
Postexposure	34.76 ± 0.13	34.88 ± 0.44	34.84 ± 0.29	34.44 ± 0.25
Neutrophils (%)				
Pre-exposure	41.60 ± 4 37	48.40 ± 4.01	52 20 ± 2.42	52.20 ± 1.46
Postexposure	48.60 ± 4.91	51.20 ± 6 72	46.80 ± 5.34	52.00 ± 2.84
Lymphocytes (%)				
Pre-exposure	55.00 ± 3.96	47.60 ± 4.81	45.40 ± 2.68	46 60 ± 1.21
Postexposure	48.80 ± 4.87	51.20 ± 6.72	52 00 ± 5.06	46.00 ± 3.15
Monocytes (%)				
Pre-exposure	4.33 ± 1.20	4.25 ± 0 75	1.80 ± 0 37	1.50 ± 0 50
Postexposure	3.67 ± 1.76	5.75 ± 0 63	2.00 ± 0.58	3.33 ± 0.33
Eosinophils (%)				
Pre-exposure	1 00 ± 0 00	1.50 ± 0.50	1.50 ± 0.50	0.00 ± 0.00
Postexposure	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0 00

[a] Mean ± S E M , N = 5

TABLE 9.1-3. MEAN[a] WHOLE BLOOD PARAMETERS FOR NZW FEMALE RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
WBC (x 10^3 cells/mm^3)				
Pre-exposure	7.20 ± 1.00	5.58 ± 0.59	6.78 ± 0 76	6.08 ± 0.51
Postexposure	7.78 ± 1.02	6.32 ± 0.88	7.02 ± 0 63	5.84 ± 0.51
RBC (x 10^6 cells/mm^3)				
Pre-exposure	6.06 ± 0.27	5.72 ± 0.12	5.50 ± 0 20	5.70 ± 0 07
Postexposure	6.23 ± 0.23	5.69 ± 0.14	5.18 ± 0.16	5.73 ± 0.17
HGB (g/dL)				
Pre-exposure	13.08 ± 0.29	12.53 ± 0.34	12.34 ± 0 27	12.42 ± 0 14
Postexposure	13.40 ± 0.46	12.50 ± 0.22	12.08 ± 0 31	12.80 ± 0.25
HCT (%)				
Pre-exposure	39 65 ± 1.17	37.28 ± 0 93	36.24 ± 0.95	37.22 ± 0 25
Postexposure	39.82 ± 1.23	36.76 ± 0.69	33.58 ± 1.44	37.30 ± 0.69
MCV (mm^3)				
Pre-exposure	65.48 ± 1.35	65.13 ± 1.13	64.48 ± 1.19	65.20 ± 1.07
Postexposure	63.96 ± 1.32	64.54 ± 0.76	64.66 ± 1.25	65.12 ± 1.22
MCH (10^{-12}/cells/mm^3)				
Pre-exposure	21.68 ± 0.58	21.95 ± 0.37	22.00 ± 0 55	21.80 ± 0.37
Postexposure	21 56 ± 0.57	21.98 ± 0.42	23.44 ± 0.98	24.40 ± 2.15
MCHC (%)				
Pre-exposure	32.98 ± 0.38	33.45 ± 0.21	34.06 ± 0.31	33.28 ± 0.29
Postexposure	33.66 ± 0.21	34.04 ± 0.56	36.28 ± 1.68	34.38 ± 0.17
Neutrophils (%)				
Pre-exposure	37.00 ± 5.73	56.25 ± 7.76	38.40 ± 3.59	57.00 ± 2.30
Postexposure	50.20 ± 6.94	43.00 ± 6.91	38.20 ± 3 80	38.80 ± 5.38
Lymphocytes (%)				
Pre-exposure	61.75 ± 5 85	51 50 ± 8 73	38.40 ± 3 59	41.80 ± 2 71
Postexposure	47.00 ± 7.11	54 80 ± 6.77	61.00 ± 3.81	58.80 ± 4.88
Monocytes (%)				
Pre-exposure	1.25 ± 0.25	1.00 ± 0 00	1.75 ± 0.48	1.25 ± 0.25
Postexposure	2.40 ± 0.68	1.80 ± 0.58	1 33 ± 0.33	2.20 ± 0.58
Eosinophils (%)				
Pre-exposure	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0 00	1.00 ± 0.00
Postexposure	1 00 ± 0.00	1.00 ± 0.00	0.00 ± 0 00	1.00 ± 0.00

[a] Mean ± S E M , N = 5

TABLE 9.1-4. MEAN VALUES[a] OF SERUM BIOCHEMISTRY PARAMETERS FOR MALE NZW RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 g/kg	0.50 g/kg	1.00 g/kg
BUN (mg/dL)				
Pre-exposure	18.08 ± 1.26[b]	14.50 ± 0.87	16.25 ± 1.98	15.82 ± 0.51
Postexposure	16.40 ± 0.70	13.60 ± 0.30	16.04 ± 0.89	14.88 ± 1.05
Creatinine (mg/dL)				
Pre-exposure	1.23 ± 0.08[b]	0.94 ± 0.04	1.00 ± 0.07	1.16 ± 0.06
Postexposure	0.66 ± 0.05	0.80 ± 0.05	1.00 ± 0.04	0.98 ± 0.05
Phosphorous (mg/dL)				
Pre-exposure	6.99 ± 0.24[b]	6.76 ± 0.10	6.63 ± 0.03	7.88 ± 1.05
Postexposure	6.29 ± 0.11	6.01 ± 0.27	5.49 ± 0.51	5.88 ± 0.23
Calcium (mg/dL)				
Pre-exposure	14.83 ± 0.33[b]	14.26 ± 0.39	14.90 ± 0.32	15.38 ± 0.32
Postexposure	14.24 ± 0.26	14.70 ± 0.34	14.88 ± 0.16	15.04 ± 0.21
Total protein (g/dL)				
Pre-exposure	6.00 ± 0.11[b]	5.92 ± 0.08	6.00 ± 0.09	6.07 ± 0.15
Postexposure	5.25 ± 0.45	5.99 ± 0.16	5.70 ± 0.42	6.08 ± 0.20
Alk. phos. (IU/L)				
Pre-exposure	275.00 ± 28.59[b]	242.40 ± 12.89	209.75 ± 31.11	220.80 ± 18.19
Postexposure	175.20 ± 18.91	247.00 ± 22.35	269.80 ± 55.16	213.60 ± 25.51
SGOT (IU/L)				
Pre-exposure	41.75 ± 20.22[b]	29.80 ± 2.08	36.75 ± 5.94	33.00 ± 5.38
Postexposure	50.20 ± 1.66	43.40 ± 6.80	29.60 ± 2.11	34.40 ± 4.34
SGPT (IU/L)				
Pre-exposure	14.00 ± 4.00[c]	27.75 ± 6.42	125.00 ± 95.00[c]	19.20 ± 5.03
Postexposure	28.20 ± 3.41	19.80 ± 4.47	15.75 ± 3.79	22.50 ± 5.07
LDH				
Pre-exposure	----------[d]	226.00 ± 21.00	74.00 ± 0.00	----------[d]
Postexposure	98.50 ± 25.96	168.60 ± 46.98	191.00 ± 29.41	89.20 ± 18.66

[a] Mean ± S.E.M., N = 5 except as noted
[b] N = 4
[c] N = 2; one value was extreme (220 IU/L) and no technical problems could be associated with this result
[d] Insufficient data

TABLE 9.1-5. MEAN VALUES[a] OF SERUM BIOCHEMISTRY PARAMETERS FOR FEMALE NZW RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 g/kg	0.50 g/kg	1.00 g/kg
BUN (mg/dL)				
Pre-exposure	20.94 ± 1 06	18 96 ± 1 72	18 32 ± 0 84	18 04 ± 1 18
Postexposure	18.34 ± 1.37	18.58 ± 1 77	19 50 ± 0 72	18 48 ± 0 78
Creatinine (mg/dL)				
Pre-exposure	0.90 ± 0.09	1 18 ± 0 05	1 10 ± 0 00	1.15 ± 0.03[b]
Postexposure	1.12 ± 0 08	1.12 ± 0 04	0.98 ± 0 02	1 06 ± 0.05
Phosphorous (mg/dL)				
Pre-exposure	6.38 ± 0.41	6 82 ± 0.17	6 43 ± 0 42	6.48 ± 0.23
Postexposure	5.56 ± 0.51	5.86 ± 0.20	5 98 ± 0 25	5.40 ± 0.21
Calcium (mg/dL)				
Pre-exposure	15.14 ± 0.12	15.70 ± 0.23	15 50 ± 0 21	15.48 ± 0.20
Postexposure	14.00 ± 0 48	13.82 ± 0.17	13.75 ± 0 75	----------[d]
Total protein (g/dL)				
Pre-exposure	6.12 ± 0.17	6 06 ± 0 12	6.08 ± 0.09	6.27 ± 0.14
Postexposure	6 38 ± 0.25[b]	5.80 ± 0.10[c]	5.98 ± 0.08	----------[d]
Alk. phos. (IU/L)				
Pre-exposure	219.00 ± 13.83	228.00 ± 7.26	284.60 ± 27.51	262.20 ± 42.96
Postexposure	180.60 ± 9 11	198.60 ± 8.68	257.00 ± 44.91	229.80 ± 29.27
SGOT (IU/L)				
Pre-exposure	48.80 ± 6.53	36.00 ± 2.43	40.25 ± 4 57[b]	39.20 ± 8.70
Postexposure	32.40 ± 2.16	33.40 ± 6.50	32.40 ± 4 25	37.00 ± 12.08
SGPT (IU/L)				
Pre-exposure	54.00 ± 8 26[b]	66.00 ± 12.15[b]	53.75 ± 13.22[b]	46.40 ± 12.79
Postexposure	42.60 ± 11.04	40.60 ± 10.20	28.40 ± 4.59	26.20 ± 5 57
LDH				
Pre-exposure	103.67 ± 32.63[c]	179.00 ± 30.28[b]	216.00 ± 10 79[b]	231 40 ± 20.47
Postexposure	129 00 ± 17 64	117.40 ± 20.23	149 40 ± 18.45	217 00 ± 20 72

[a] Mean ± S E M , N = 5
[b] N = 4
[c] N = 3
[d] Insufficient data.

TABLE 9.1-6. MEAN ORGAN WEIGHTS[a] (g) AND ORGAN-TO-BODY WEIGHT RATIOS[b] OF MALE NZW RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Organ	Control[c]	0.25 g/kg[d]	0.50 g/kg[c]	1.00 g/kg[d]
Adrenals	0.22 ± 0.02	0 21 ± 0.01	0 22 ± 0 01	0 21 ± 0 02
Ratio	0.01 ± 0.00	0.01 ± 0 00	0 01 ± 0 00	0 01 ± 0.00
Brain	8.93 ± 0.11	9 16 ± 0 21	9 16 ± 0 23	9.05 ± 0.19
Ratio	0.30 ± 0.01	0 29 ± 0 01	0 31 ± 0 01	0.30 ± 0.01
Heart	8.84 ± 0.61	8.99 ± 0 60	9.56 ± 0 65	9 26 ± 0.75
Ratio	0.30 ± 0.02	0.29 ± 0.02	0.32 ± 0.02	0.31 ± 0.02
Kidney	17.50 ± 0.88	20.26 ± 1 14[e]	19.30 ± 0.74	19.05 ± 0.82
Ratio	0.59 ± 0.02	0.64 ± 0 03[e]	0.65 ± 0.02[e]	0.63 ± 0.01
Liver	96.83 ± 3.29	123 40 ± 8 93[e]	123.52 ± 7.87[e]	117.81 ± 6.87
Ratio	3.27 ± 0.09	3.92 ± 0 23	4.14 ± 0.20[e]	3.89 ± 0.18
Lung	25 08 ± 2.61	25.90 ± 2 62	27.93 ± 3.16	27.97 ± 2.83
Ratio	0.85 ± 0.10	0 84 ± 0 10	0.95 ± 0.12	0.94 ± 0.11
Testes	3.38 ± 0.17	3.39 ± 0 27	3.33 ± 0 27	3.64 ± 0.24
Ratio	0.11 ± 0.00	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
Spleen	0.93 ± 0.05	0.99 ± 0.10	0.90 ± 0.08	0.96 ± 0.07
Ratio	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Thymus	4.13 ± 0.33	4.32 ± 0.39	4.17 ± 0.33	2.55 ± 0.75[e]
Ratio	0.14 ± 0.001	0.14 ± 0.01	0.14 ± 0.01	0.08 ± 0.02[e]
Whole body (kg)	2.96 ± 0.07	3.14 ± 0.09	2.97 ± 0 08	3.02 ± 0.08

[a] Mean ± S E M
[b] Organ weight/body weight x 100
[c] N = 10
[d] N = 9
[e] Significantly different from controls at $p < 0.05$ level

TABLE 9.1-7. MEAN ORGAN WEIGHTS[a] (g) AND ORGAN-TO-BODY WEIGHT RATIOS[b] OF FEMALE NZW RABBITS FOLLOWING 21-DAY REPEATED DERMAL EXPOSURE TO CYCLOTRIPHOSPHAZENE

Organ	Control	0.25 g/kg	0.50 g/kg	1.00 g/kg
Adrenals	0.24 ± 0.01	0 26 ± 0.02	0.21 ± 0.01	0.26 ± 0.02
Ratio	0.01 ± 0.00	0.01 ± 0 00	0 01 ± 0 00	0.01 ± 0 00
Brain	8.79 ± 0.11	8 98 ± 0 18	8.84 ± 0 13	8.69 ± 0 19
Ratio	0.30 ± 0.01	0 28 ± 0.01	0.28 ± 0 01	0.29 ± 0.01
Heart	8.40 ± 0.54	10.04 ± 0.66	8.84 ± 0 59	8.92 ± 0.57
Ratio	0.29 ± 0.02	0.31 ± 0.02	0.28 ± 0.02	0.29 ± 0.02
Kidney	16.55 ± 0.82	19.92 ± 0.52[c]	18.50 ± 0.59	17.88 ± 0.83
Ratio	0.56 ± 0.02	0.61 ± 0.02[c]	0.59 ± 0.02	0.59 ± 0.02
Liver	107.69 ± 4.14	123.57 ± 6.31[c]	118.74 ± 7.66[c]	109.89 ± 7.50
Ratio	3.64 ± 0.09	3.79 ± 0.13	3.79 ± 0.21	3.61 ± 0.19
Lung	26.44 ± 2.62	24.46 ± 1.73	28.71 ± 2.90	32.60 ± 1.43
Ratio	0.90 ± 0.09	0.76 ± 0.06	0.92 ± 0.09	1.08 ± 0.05
Ovaries	0.21 ± 0.02	0.22 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
Ratio	0.01 ± 0.00	0.01 ± 0 00	0.01 ± 0.00	0.01 ± 0.00
Spleen	0.99 ± 0.07	1 10 ± 0.08	1.02 ± 0.07	1.12 ± 0.05
Ratio	0.03 ± 0.00	0.03 ± 0.00	0 03 ± 0.00	0.04 ± 0.00
Thymus	3.44 ± 0.18	4.30 ± 0.30	3.96 ± 0.36	3.96 ± 0.24
Ratio	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Whole body (kg)	2.95 ± 0.06	3.25 ± 0.07	3.13 ± 0.06	3.03 ± 0.09

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from controls at $p < 0.05$ level

9.2 DETERMINATION OF THE TOXICITY OF CYCLOTRIPHOSPHAZENE HYDRAULIC FLUID BY 21-DAY REPEATED INHALATION EXPOSURE

E.R. Kinkead, B.T. Culpepper, S.S. Henry, E.C. Kimmel, and C.R. Doarn

INTRODUCTION

The Navy has developed candidate hydraulic fluids with chemical structures of cyclotriphosphazene (CTP) cyclic esters. Acute toxicity studies demonstrated that this hydraulic fluid is nontoxic by oral or dermal administration (unreported data) Eye and skin irritation tests, as well as skin sensitization tests, proved negative (Kinkead and Bowers, 1985). The hydraulic fluid was not detected in the blood or urine of rats following exposure by aerosol inhalation or dermal contact (unreported data). The oral LD_{50} of tolyltriazole, an additive to this hydraulic fluid, in rats is 675 mg/kg (Huntingdon Research Center Report, 1972)

This report addresses the effects of repeated inhalation exposure and was designed to measure the toxic effects associated with repeated exposure to CTP over a limited time. An additional objective was to determine a "no observed effect" exposure concentration of the compound These studies were not designed to identify those effects that have a long latency period (e.g., carcinogenicity, decreased life expectancy). The selected species and group size used in this study conformed to the U S. Environmental Protection Agency's Health Effects Test Guidelines (1982).

MATERIALS AND METHODS

Test Agent

The CTP cyclic ester hydraulic fluid contained 0.1% tolyltriazole. It was supplied by the Toxicology Detachment of the Naval Medical Research Institute (NMRI/TD), Wright-Patterson Air Force Base, OH. The pertinent data on these materials are provided below

CTP ester:

NMRI/TD No	4341-1
Vapor Pressure, mmHg	
65°C	0 49
149°C	12.0
Specific Gravity (g/mL)	1.445

Tolyltriazole:

Chemical Formula	$C_7H_7N_3$
CAS No.	29385-43-1
Synonyms	Methylbenzotriazole
	1,2,3-Triazole
	(methylphenyl)

This hydraulic fluid is a mixture of parent compound isomers including dimers, trimers, and tetramers of cyclotriphosphazene. The approximate molecular weight of the fluid is 1000 g/mole.

Test Agent Quality Control

A Varian 3700 gas chromatograph (GC) equipped with a flame ionization detector and a 50-meter, 5% phenylmethyl silicone capillary column was utilized in conjunction with a Hewlett-Packard 3388 computing integrator to measure peak area and record chromatograms of the test material. GC profiles were obtained of the material as received, as aerosolized, and as residue from the nebulizer system.

Animals

Male and female Fischer 344 (F-344) rats, 9 to 11 weeks of age at the study onset, were purchased from Charles River Breeding Labs (Kingston, NY). Upon receipt, the animals were randomized by a simple random sampling of animal identification numbers over each treatment and control group. All rats were judged to be in good health following a two-week quarantine period. Prior to the study, rats were group-housed (two to three per cage) in clear plastic cages with wood chip bedding. During the study, the rats were housed individually in assigned exposure cage locations. Each exposure day, the exposure cages were rotated one position in a clockwise direction within the inhalation chambers. Water and feed (Purina Formulab #5008) were available *ad libitum* except during the exposures and 10 h prior to sacrifice. Rats were maintained on a 12-h light/dark cycle.

METHODS AND EXPERIMENTAL EVALUATIONS

A detailed description of the methods and experimental evaluations performed for this inhalation toxicity study has been published (Kinkead et al , 1988).

RESULTS

Chamber Analysis

The composition of the test material prior to aerosolization was compared to both the generator residues and the aerosol samples collected on filters. The composition of the generator

material did not change during the course of the 6-h study and the aerosol generated contained the same mixture of components as the starting material. The vapor content of the chamber was below the detection limits of the analytical methods employed

The specified nominal concentrations of 0 25, 0 5, and 1.0 mg CTP/L were maintained during the three-week exposure period All daily mean chamber concentrations were within 10% of the desired concentration except for exposure Day 13 when the mean daily concentration was 84% of nominal. Examination of particle size data indicated that the aerosols produced in all exposure chambers were of a respirable size. A summary of the measured daily mean chamber concentrations and particle size analyses has been reported (Kinkead et al , 1988).

Biological Data

A total of 80 F-344 rats were included in the three-week inhalation toxicity study. There were no deaths resulting from the exposures. No behavioral or physical signs of toxic stress were observed during the exposures. Despite the random assignments to dose groups, there were statistical differences between the mean group weights of male rats at the start of the exposures Therefore, mean weight gains and losses were analyzed Weight gains of treated groups were significantly depressed when compared to the respective control group of the same sex following one week of exposure. However, after the first week, weights (gains/losses) were similar among groups of the same sex (Table 9.2-1).

TABLE 9.2-1. MEAN BODY WEIGHTS[a] OF F-344 RATS DURING 21-DAY INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE HYDRAULIC FLUID

		Body Weights (g)			
Sex	Dose Group	Day 0	Day 7	Day 14	Day 21[b]
Male	Control	199 ± 6.2	226 ± 1.8	240 ± 2.0	234 ± 2.3
	0.25 mg/L	194 ± 4.9[c]	213 ± 2.1[d,e]	228 ± 2.8[d]	222 ± 2.7[d]
	0.50 mg/L	210 ± 2.7[d]	215 ± 2.7[d,e]	229 ± 3.0[d]	223 ± 3.1[d]
	1.00 mg/L	199 ± 3.5	207 ± 2.7[d,e]	223 ± 2.9[d]	218 ± 2 5[d]
Female	Control	147 ± 1.8	151 ± 1 6	157 ± 1 8	147 ± 2.2
	0.25 mg/L	151 ± 1.4	148 ± 1.6	153 ± 1.4	146 ± 1.5
	0.50 mg/L	147 ± 2.1	144 ± 2.3[c,f]	148 ± 2.7[d]	143 ± 2.5
	1.00 mg/L	148 ± 2 6	145 ± 2 7[c,f]	151 ± 2.4[c]	146 ± 2.6

[a] Mean ± S E M , N = 10
[b] Fasted weights
[c] Significantly different from control at $p < 0\ 05$ using two factorial analysis of variance corrected for multiple comparisons
[d] Significantly different from control at $p < 0\ 01$ using two factorial analysis of variance corrected for multiple comparisons
[e] Seven day weight gain significantly less than controls at $p < 0\ 01$ using two factorial analysis of variance corrected for multiple comparisons
[f] Seven day weight gain significantly less than controls at $p < 0\ 05$ using two factorial analysis of variance corrected for multiple comparisons

None of the clinical chemistry parameters evaluated revealed significant exposure effects on the rats (Tables 9.2-2 and 9.2-3). Analysis of hematology parameters (Tables 9.2-4 and 9 2-5) revealed no statistical differences between test and control groups, and all group means for these parameters were within the normal range for the age and species of test animals used (Wolford et al., 1986)

TABLE 9.2-2. MEAN VALUES[a] OF SERUM BIOCHEMISTRY PARAMETERS FOR MALE F-344 RATS FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
BUN (mg/dL)	16.9 ± 0.6	16 5 ± 0.4	17.9 ± 0.7	16.9 ± 0.5
Creatinine (mg/dL)	0.6 ± 0.0	0.6 ± 0 0	0.6 ± 0.0	0.6 ± 0.0
Calcium (mg/dL)	10.7 ± 0.2	10.7 ± 0 2	10.7 ± 0.1	10.6 ± 0.2
Total protein (g/dL)	7.5 ± 0.1[b]	7.4 ± 0.1[b]	7.3 ± 0.1[c]	7.5 ± 0.1[b]
Alk. phos. (IU/L)	177.1 ± 5.6	174.7 ± 5.3	182.1 ± 8.7	185 7 ± 10.2
LDH (IU/L)	826.8 ± 110.4	596.9 ± 52.7	712 5 ± 95.4	625.3 ± 99.3

[a] Mean ± S E M
[b] N = 7
[c] N = 6

TABLE 9.2-3. MEAN VALUES[a] OF SERUM BIOCHEMISTRY PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
BUN (mg/dL)	21.7 ± 0.7	22.5 ± 1.2	21.1 ± 0.7	22 3 ± 0.7[b]
Creatinine (mg/dL)	0.6 ± 0.0	0.6 ± 0 0	0.6 ± 0.0	0.6 ± 0.0[b]
Calcium (mg/dL)	10.7 ± 0.2	10 3 ± 0.2	10 1 ± 0.2	9.9 ± 0.2[c]
Total Protein (g/dL)	7.1 ± 0.1	6.9 ± 0.3	7 0 ± 0.0	7.1 ± 0 1[c]
Alk. Phos. (IU/L)	124.1 ± 4.1	125 8 ± 9 2	128.7 ± 9.0	125.5 ± 3.5[c]
LDH (IU/L)	584 0 ± 125 3	465.3 ± 90.7	745 6 ± 135 5	783 1 ± 149 8[b]

[a] Mean ± S E M
[b] N = 9
[c] N = 8

TABLE 9.2-4. MEAN[a] WHOLE BLOOD PARAMETERS FOR MALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
WBC (x 10^3 cells/mm^3)	8 00 ± 0.51	7.66 ± 0.35	7 60 ± 0 34	7.16 ± 0.53
RBC (x 10^6 cells/mm^3)	8 56 ± 0.14	8 46 ± 0.09	8 63 ± 0 07	8.18 ± 0.12
HGB (g/dL)	16.99 ± 0.19	16 63 ± 0 10	16 71 ± 0 12	16.22 ± 0 23
HCT (%)	45 48 ± 0.75	44.53 ± 0 60	45.31 ± 0.46	42 88 ± 0 58
MCV (fl)	52 99 ± 0.34	52 52 ± 0.41	52 48 ± 0.25	52.44 ± 0.31
MCH (pg)	19.89 ± 0.17	19 68 ± 0 17	19 39 ± 0.15	19.85 ± 0.15
MCHC (%)	37.50 ± 0.27	37.45 ± 0.39	36 90 ± 0.23	38.02 ± 0.31
Neutrophils (%)	27 30 ± 2 68	20 40 ± 1 82	22 40 ± 1.66	25 50 ± 1.71
Lymphocytes (%)	68.10 ± 2.88	77 10 ± 1 59	75 20 ± 2 00	70.80 ± 1.70
Monocytes (%)	2.38 ± 0.46	2 00 ± 0 70	2 33 ± 0 33	2.17 ± 0 40
Eosinophils (%)	1 20 ± 0 20	1 17 ± 0.17	1 17 ± 0 17	2 00 ± 0 58
Atypical lymphocytes (%)	2 25 ± 0 31	1 25 ± 0 16	1 60 ± 0 60	1 88 ± 0 40

[a] Mean ± S E M , N = 10

TABLE 9.2-5. MEAN[a] WHOLE BLOOD PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Parameter	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
WBC (x 10^3 cells/mm^3)	9.72 ± 0.93	10.94 ± 1.22	11 36 ± 1 19	10 77 ± 0.68
RBC (x 10^6 cells/mm^3)	7.87 ± 0.15	7.72 ± 0.11	7 63 ± 0 10	7.64 ± 0 09
HGB (g/dL)	16.40 ± 0.30	15.85 ± 0.20	15 74 ± 0 17	15.82 ± 0 17
HCT (%)	42.72 ± 0 88	41.74 ± 0.60	41 44 ± 0 48	41 35 ± 0.51
MCV (fl)	54.19 ± 0 28	54 14 ± 0 37	54 23 ± 0 41	54.07 ± 0.21
MCH (pg)	20 86 ± 0.14	20.55 ± 0.22	20 65 ± 0.17	20 53 ± 0.22
MCHC (%)	44.00 ± 5 69	38.26 ± 0.20	38 00 ± 0 35	38 03 ± 0 34
Neutrophils (%)	19.78 ± 1.98	21.00 ± 1 69	20 63 ± 2 58	21.30 ± 2.45
Lymphocytes (%)	78.00 ± . 18	75.20 ± 1 95	76.50 ± 2 75	74.20 ± 2.33
Monocytes (%)	1.38 ± 0 18	3 00 ± 0.91	2.00 ± 0 31	2.75 ± 0.73
Eosinophils (%)	1.00 ± 0.00	1.71 ± 0.47	1 00 ± 0 00	2.11 ± 0.20
Atypical lymphocytes (%)	2.50 ± 0.50	2.50 ± 0 65	0.00 ± 0 00	1 00 ± 0.00

[a] Mean ± S E M , N = 10

Organ weights and organ-to-body weight ratios measured at necropsy (Tables 9.2-6 and 9.2-7) identified the spleen, liver, and testes of male exposed rats as significantly different from their respective controls. Statistically significant decreases in absolute spleen weights in the 0.25 and 1.0 mg CTP/L groups were not confirmed by comparisons of spleen-to-body weight ratios. The liver-to-body weight ratios of all test groups of both sexes were slightly higher than their respective controls; however, the increases were statistically significant only in the 0 5 mg CTP/L concentration group of male rats and 1 0 mg CTP/L of female rats. Testes-to-body weight ratios were significantly higher than controls ($p < .01$) for all three exposure groups

Gross pathological examination of the animals at necropsy failed to reveal any dose-related lesions Light microscopy revealed pulmonary alveolar macrophages in 100% of the high dose animals with percentages decreasing through the mid and low dose groups Fifty percent fewer control animals exhibited alveolar macrophages (alveolar histiocytosis). Statistical analysis of lesion severity indicated significant differences in alveolar histiocytosis between the high dose male and female rats and their corresponding control group ($p < 0.01$)

Hyaline droplets were seen in the kidneys of CTP exposed animals (100, 100, and 50% for males and 80, 80, and 70% for females at 1 0, 0.5, and 0 25 mg CTP/L, respectively), while none were seen in the kidneys of control animals Statistical analysis of hyaline droplet severity indicated significant differences between the male rats in the 0 25 mg/L group ($p < 0 05$) and the two higher concentration groups ($p < 0 01$) and their corresponding control group, and between female rats in each CTP exposure group and their corresponding control group ($p < 0 01$)

DISCUSSION

Repeated inhalation of this hydraulic fluid resulted in a transitory depression in body weight gains in both male and female treated rats during the first week of exposure Body weight gains of

the treated rat groups were comparable to their respective control groups during the final two weeks of exposure. Although an increase occurred in the gross liver weights of the high concentration female rats, no lesions were observed when the liver tissue was examined by light microscopy.

Based on the analysis of incidence and severity data, the only CTP-related effects were pulmonary alveolar histiocytosis, which was most severe in high dose male and female rats, and renal tubule hyaline droplet accumulation. Although there was a high incidence of hyaline droplet accumulation in the renal tubule epithelium of male and female rats, these lesions were minimal to mild, and this is a common background lesion, especially in male rats. Nevertheless, the differences from controls, which lacked renal hyaline droplet accumulation, suggested that CTP exposure may trigger hyaline droplet accumulation. The increased lung macrophages were not associated with detectable injury to lung structure and were most likely associated with a pulmonary clearance response. Preliminary data indicates that CTP is phagocytized readily by lavaged rat alveolar macrophages that are maintained *in vitro* (Dr C.S. Godin, personal communication). The apparent mildness of the lung and renal lesions suggests that CTP had little toxicity in rats at the concentrations tested.

TABLE 9.2-6. MEAN ORGAN WEIGHTS[a] (g) AND ORGAN-TO-BODY WEIGHT RATIOS[b] OF MALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Organ	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
Adrenals	0.07 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.00
Ratio	0.03 ± 0.00	0.04 ± 0.01	0 03 ± 0 00	0.03 ± 0.00
Brain	1.73 ± 0.03	1.76 ± 0 03	1.98 ± 0.17	1.73 ± 0 03
Ratio	0.74 ± 0.01	0.79 ± 0.02	0.89 ± 0.08	0.80 ± 0.01
Heart	0.82 ± 0.02	0.82 ± 0.03	0.80 ± 0.02	0.79 ± 0.02
Ratio	0.35 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.36 ± 0.01
Kidney	1.84 ± 0.03	1.77 ± 0.04	1.80 ± 0.04	1.76 ± 0.04
Ratio	0.79 ± 0.01	0 80 ± 0.01	0 81 ± 0.01	0.81 ± 0 01
Liver	7.83 ± 0.17	7.53 ± 0.19	7 79 ± 0.19	7 30 ± 0.52
Ratio	3.34 ± 0.06	3.39 ± 0.05	3.50 ± 0.05[c]	3.35 ± 0 23
Lung	1.62 ± 0.07	1.44 ± 0.04	1.72 ± 0.18	1 54 ± 0.05
Ratio	0.69 ± 0.03	0 65 ± 0 02	0.77 ± 0.08	0.71 ± 0.02
Testes	2.95 ± 0.03	2.97 ± 0 01	2.93 ± 0.05	2.92 ± 0.04
Ratio	1.26 ± 0.01	1 34 ± 0.02[d]	1.32 ± 0.02[d]	1.34 ± 0 01[d]
Spleen	0.54 ± 0.02	0.48 ± 0 01[c]	0.51 ± 0.02	0.47 ± 0.01[d]
Ratio	0.23 ± 0.01	0.22 ± 0 01	0.23 ± 0.01	0.22 ± 0.01
Thymus	0.32 ± 0 02	0 29 ± 0 03	0.33 ± 0.02	0.30 ± 0 02
Ratio	0.14 ± 0.01	0.13 ± 0 01	0.15 ± 0.01	0.14 ± 0 01
Whole body	234 10 ± 2 25	222.10 ± 2.74[d]	222 70 ± 3.10[d]	217 80 ± 2 54[d]

[a] Mean ± S E M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from controls at $p < 0.05$ level
[d] Significantly different from controls at $p < 0.01$ level

TABLE 9.2-7. MEAN ORGAN WEIGHTS[a] (g) AND ORGAN-TO-BODY WEIGHT RATIOS[b] OF FEMALE F-344 RATS FOLLOWING 21-DAY INHALATION EXPOSURE TO CYCLOTRIPHOSPHAZENE

Organ	Control	0.25 mg/L	0.50 mg/L	1.00 mg/L
Adrenals	0.07 ± 0.01	0.07 ± 0.00	0 07 ± 0.00	0.07 ± 0.00
Ratio	0.05 ± 0.01	0.05 ± 0 00	0.05 ± 0.00	0 05 ± 0.00
Brain	1.72 ± 0.02	1.74 ± 0 02	1.69 ± 0.03	1 71 ± 0 03
Ratio	1.17 ± 0.02	1.19 ± 0.01	1 18 ± 0.03	1 18 ± 0.03
Heart	0.59 ± 0 01	0.59 ± 0.02	0.58 ± 0.01	0.58 ± 0.01
Ratio	0.40 ± 0.01	0.40 ± 0.01	0.40 ± 0.01	0.40 ± 0.01
Kidney	1.17 ± 0.02	1.20 ± 0 02	1.19 ± 0.03	1.17 ± 0.03
Ratio	0.79 ± 0.01	0 82 ± 0 01	0.83 ± 0 02	0 80 ± 0.02
Liver	4.31 ± 0.10	4.69 ± 0 10	4.73 ± 0.11	4 90 ± 0 09
Ratio	2.93 ± 0 03	3 21 ± 0 04	3.30 ± 0.06	3.38 ± 0.06[c]
Lung	1 16 ± 0.02	1.18 ± 0.03	1.16 ± 0.02	1.29 ± 0.03
Ratio	0.79 ± 0.01	0.81 ± 0.02	0.81 ± 0.02	0.89 ± 0.03
Ovaries	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.00	0.15 ± 0.02
Ratio	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.01
Spleen	0.40 ± 0.01	0.41 ± 0 02	0.38 ± 0.01	0.41 ± 0.01
Ratio	0.27 ± 0.00	0.28 ± 0 01	0.27 ± 0.01	0.28 ± 0.01
Thymus	0.30 ± 0.02	0.30 ± 0 02	0.28 ± 0.01	0.30 ± 0.01
Ratio	0.21 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.21 ± 0.01
Whole body	234.10 ± 2.19	222.10 ± 1.47	222.70 ± 2.51	217.80 ± 2.62

[a] Mean ± S E.M , N = 10
[b] Organ weight/body weight x 100
[c] Significantly different from controls at $p < 0.05$ level

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9.3 DETERMINATION OF THE REPEATED ORAL TOXICITY OF HALOCARBON OIL, SERIES 27-S

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INTRODUCTION

Halocarbon oil, Series 27-S (HC 27-S) is a mixture of chlorotrifluoroethylene (CTFE) oligomers It is used as a lubricating oil for pumps in hyperbaric chambers by the U S Navy Sea Systems Command. In addition, HC 27-S is used to lubricate all O-rings on doors and service locks of the hyperbaric chambers.

CTFE, a mixture of chlorotrifluoroethylene oligomers similar to HC 27-S, is metabolized in part to inorganic fluoride which is excreted in the urine. Plasma and urine fluoride levels in Fischer 344 (F-344) rats remained elevated for more than a week following oral exposure to CTFE and for at least 24 h following inhalation exposure Neither plasma nor urine fluoride levels were elevated following dermal exposure (Kinkead et al., 1987). A recent 90-day inhalation study has shown that CTFE causes hepatomegaly in male and female rats (see Section 3 6 of this report.)

The rat was selected as the test species to minimize space requirements and to allow comparison to the above-mentioned studies. The numbers of animals per test group were kept to the minimum necessary for appropriate statistical analysis. These studies were performed as a preliminary assessment of the toxicity of repeated exposure to HC 27-S.

MATERIALS AND METHODS

Animals

Male F-344 rats, weighing between 180 and 220 g and females weighing between 150 and 200 g, were purchased from Charles River Breeding Labs (Kingston, NY). Quality control assessments, conducted during a two-week quarantine period, showed the animals to be in acceptable health.

The rats were housed individually except during the quarantine period and the 14-day holding period postdosing. Prior to treatment the rats were placed in Nalgene (Nalge Company, Rochester, NY) metabolism cages for a one-week acclimatization period All rats were fed Purina Formulab 5008 (food in metabolism cages was powdered) and deionized distilled water *ad libitum*, with the exception that rats were fasted for 16 h prior to oral dosing All animals were identified by toe clipping and were maintained on a light/dark cycle set at 12-h intervals.

Test Material

The HC 27-S test material was supplied by the Naval Medical Research Institute/Toxicology Detachment at Wright-Patterson Air Force Base It was manufactured by Halocarbon Products Corporation (Hackensack, NJ) to conform to U S Military Specification MIL-L-24574 The oil has a

vapor pressure of less than 0.01 mm Hg at 27°C and contains 0.1% of an unspecified organic acid rust inhibitor. This sample had a density of 1.930 g/mL at room temperature and will decompose at temperatures above 260°C.

The oligomer mixtures, HC 27-S and CTFE military hydraulic fluid, are produced from the same basic monomer, CTFE, and are distinguished by average chain length. Chromatograms for the two materials, which were evaluated under the same conditions, showed a repeating series of peaks. HC 27-S and CTFE share a common set of peaks, but the majority of the CTFE oligomers elute earlier than the HC 27-S oligomers. When the chromatograms of CTFE and HC 27-S were compared with limited carbon fraction mixtures, they revealed that the CTFE centered on the C_6 fraction while HC 27-S centered after the C_8 fraction (possibly at the C_{10}).

REPEATED ORAL ASSAY

The animals were dosed repeatedly with 2.5 g HC 27-S/kg body weight. Six groups of six F-344 rats (per sex) were dosed daily, including weekends, by gavage. Each of three test groups per sex received the same daily dose but was sacrificed at different times. Each of three control groups received an equal volume of distilled water and was sacrificed with its corresponding treatment group. The HC 27-S was administered as neat agent and the dose volumes were calculated from the individual body weights, adjusted daily. Dosing was performed prior to 0930 h. Food, provided following gavage, was removed at 1630 h each day.

Body weights were measured daily throughout the study. Water consumption and urine output were measured gravimetrically each day during the dosing period. Urine samples from one day predosing, from Days 1, 3, 5, 7, 14, and 21 during dosing, and from Day 14 postdosing were analyzed (Ames Multistix, Ames Division, Miles Laboratories, Elkhart, IN) for pH, protein, bilirubin, urobilinogen, glucose, ketone, and occult blood. In addition, specific gravity and creatinine values were assessed.

The rats were fasted 16 h prior to sacrifice. To eliminate the possible interference of Halocarbon anesthesia, carbon dioxide inhalation was used for euthanasia. One test and one control group were sacrificed on the morning following Day 7 of dosing; test and control groups were also sacrificed on the morning following Day 21 of dosing; and the final groups were sacrificed 14 days after the completion of dosing. At the time of sacrifice, blood was collected from the posterior *vena cava* for whole blood and serum analyses. The following organs were weighed: heart, pituitary, liver, spleen, thymus, kidneys, testes, ovaries, and brain. A gross pathologic examination was performed on each rat and specified tissues were collected and prepared for histopathologic examination. The right femur of each was collected for X-ray elemental analysis and scanning electron microscopy.

Fluoride Analysis

A fluoride specific ion electrode was used as described by Neefus et al (1970) to determine fluoride ion content in urine This method utilized synthetic urine as well as a buffer for standardization.

The method of Singer and Ophaug (1979) was used to determine unbound or ionic fluoride in plasma A fluoride specific electrode directly measured fluoride ion concentration following dilution of the plasma in a simple buffer system The method was standardized using known concentrations of fluoride ion in the buffer

X-Ray Elemental Analysis

X-ray elemental analysis was conducted on the right femur of three rats of each sex per group The femur was cleaned (the ends removed) and fixed in 10% neutral buffered formalin for 48 h. At midshaft, the femurs were trimmed into 7- to 10-mm lengths and were processed for scanning electron microscopy and X-ray analysis by dehydration with a graded series of alcohols (ethanol) The femurs were sealed with parafilm cylinders containing 100% alcohol, frozen in liquid nitrogen, and then fractured into two halves. After thawing in fresh 100% alcohol, the femurs were dried and mounted on aluminum stubs. For scanning electron microscopy, the fracture face was mounted in an upright position and sputter-coated with gold The femurs were mounted on their sides and a low-power scanning electron microscopy photograph documented the site of X-ray analysis. Six spectra were collected from each femur in two sets of three Spectra were collected from right to left in each set. The second set (D,E,F) was collected to the left of the first set (A,B,C). Spectra were analyzed for Ca and P using a Kevex 7000 X-ray analysis system.

Statistical Analysis

Statistical analyses of collected data were as follows: (1) a repeated multivariate analysis of variance with Ryan-Einot-Gabriel-Welsh F-tests used for comparisons (Dixon, 1985; Barcikowski, 1983) of body weights, water consumption, urine volumes, and urine and blood chemistries between control and test groups; and (2) a two-factorial analysis of variance for both sets of blood parameters, calcium-phosphorus (CaP) ratios, and organ weights (Dixon, 1985; Barcikowski, 1983). Histopathologic data were analyzed using the Yates' corrected Chi-square test (Zar, 1974). A probability of 0.05 or less denoted a significant statistical change from controls

RESULTS

Repeated Oral Assay

Three test and two control male rats and four test female rats died during the repeated dosing regimen prior to scheduled sacrifice Two test and one control male rat and two of four female rats

had sufficient gavage trauma to have been the cause of death. Tonoclonic spasms were noted in the female test rats after four days treatment and were noted sporadically throughout the rest of the study. Convulsions were observed in four different rats on three separate occasions. Not all female rats were observed to have convulsed, nor was a single rat observed to have convulsed more than once. Test subjects of both sexes had mild diarrhea by five to six days and appeared lethargic by eight to 10 days. The female rats appeared unkempt and kyphotic by 11 days into the study. A few treated rats had dried blood and/or hematoporphyrin-like residue around the mouth and nares. Convulsions were not noted in male rats at any time throughout the study.

Both sexes of rats dosed with HC 27-S had lower mean body weights than their respective control group at most weighing dates. Following an early depression in the mean body weight of the male test group (Days 1 and 3), no difference from the controls was noted until Day 17, after which the mean body weights of the test group were significantly ($p<0.01$) depressed throughout the remainder of the study. The mean body weight of the female test group was generally lower than the control group but the difference was statistically significant only on Days 3 through 17.

The mean daily water consumption of treated animals was significantly less ($p<0.01$) throughout the 21-day treatment period. Overall, the treated male rats consumed 17% less water than their respective controls while the test female animals consumed 25% less water than the controls. Commensurate with the decrease in daily water consumption was a decrease in urine output in both sexes of rats. Urine output by both male and female test animals was generally depressed ($p<0.01$) throughout the treatment period. Based on the days when urine volumes were measured, male treated animals produced 75% of the urine volume of control males while the female treatment group produced only 54% of the urine volume of control females.

The urine clinical parameters analyzed periodically during the study were generally within normal limits. The only parameter that indicated an apparent treatment-related effect was creatinine which increased after a single treatment and gradually returned toward control values. Serum glutamic oxalacetic transaminase (SGOT) and lactate dehydrogenase (LDH) values in female rats were the only urinary biochemistry parameters showing treatment-related effects. The SGOT activity of the female test animals was higher ($p < 0.01$) at five of the seven time points assessed during the treatment period; however, the enzyme activity returned toward control values during the 14-day post-treatment period. The LDH concentrations in the urine of both control and treated female rats generally increased throughout the study. However, the increase was more marked in the treated animals. The LDH content of their urine was almost twice (189%) that of the controls at the 35-day sacrifice.

There was a significant decrease in serum glucose values in the test male rats at each evaluation period while female test animals had increased ($p<0.01$) glucose concentrations at 21 and 35 days. Both sexes had increased ($p<0.05$) albumin concentrations at 21 and 35 days, but only the females had a commensurate increase in total protein at those evaluation periods. Several parameters in male rats were outside normal ranges and appeared to be treatment related. Blood urea nitrogen (BUN) concentrations were 28 and 78% greater ($p<0.05$) than control concentrations at 21 and 35 days, respectively. Serum concentrations of alkaline phosphatase of the treated animals progressively increased ($p<0.01$) by 35, 179, and 216% greater than control concentrations at each of the evaluation periods. Albumin concentrations were also greater ($p<0.05$) at each of the evaluation periods; however, the increases above control values were not as dramatic (8, 25, and 21%, respectively, at the 7-, 21-, and 35-day evaluations).

Inorganic urinary fluoride concentrations, measured periodically during the study, increased throughout the treatment period, especially during the first seven days. The fluoride concentrations in the urine returned toward control levels following treatment; however, complete recovery was not achieved by 14 days post-treatment. The increase in urine fluoride concentrations was statistically significant after a single treatment in both male and female rats. However, plasma inorganic fluoride concentrations of the treated rats, measured at each sacrifice, were not elevated above control values.

X-ray elemental analysis was performed on femurs from three rats per sex per group following sacrifice to determine if deposition of fluoride in the bone matrix was sufficient to alter CaP ratios. Treated male rats had CaP ratios that were significantly greater than the ratios of the control male rats at each sacrifice period (Table 9.3-1). No CaP ratio differences were found between treated and control female rats at any of the sacrifice periods.

Liver and kidney and a number of other organ weight means and organ-to-body weight ratio means of treated rats were statistically different ($p<0.05$) than the controls. Except for liver and kidney, however, no remarkable trends were noted for these organs and the changes were not considered treatment related (Table 9.3-2). Increases in relative kidney weights of the HC 27-S-treated rats of both sexes occurred at all of the sacrifice periods. The increased kidney-to-body weight ratio of female rats remained consistent throughout the study, while the ratio in male animals continued to increase during the treatment and post-treatment period. Mean liver weights of the treated rats were markedly increased over controls except at the initial (seven-day) sacrifice of female rats. Relative liver weights of the HC 27-S treated male rats were increased over controls by 70, 175, and 180% at the 7, 21, and 35 days sacrifice periods, respectively, and those of the female treated rats were increased over controls by 35, 125, and 65% at the respective sacrifice periods. The relative liver weights of treated male rats stabilized between sacrifice Days 21 and 35 after treatment with HC 27-S

had been terminated An increase ($p<0.05$) in the relative testes weights of the treated male rats was noted at the 21- and 35-day sacrifice periods However, the absolute weight of the testes increased at rates comparable to that of controls throughout the study

TABLE 9.3-1. MEAN[a] CaP RATIOS OF FEMURS FROM RATS FOLLOWING REPEATED ORAL ADMINISTRATION OF HC 27-S (N = 3)

Sex/Group	7 Days	21 Days	35 Days
Males			
Control	1.63 ± 0.02	1 64 ± 0.02	1.55 ± 0 03
Test	2.05 ± 0 02[b]	1.96 ± 0 02[b]	1.87 ± 0.06[b]
Females			
Control	1.63 ± 0.02	1 59 ± 0 01	1.68 ± 0 01
Test	1.63 ± 0.03	1.66 ± 0 02	1.68 ± 0.03

[a] Mean ± S E M (N)
[b] Statistically different from controls at $p < 0.01$ using a two factorial analysis of variance

Gross pathologic findings in rats from each sacrifice consisted of enlarged livers and adrenal glands in both sexes. Enlarged mesenteric lymph nodes were noted in female rats killed after seven days. Generalized atrophy of the thymus was noted in a number of the male rats killed at 21 and 35 days.

Significant microscopic changes were restricted to the organs of deglutition (pharynx/larynx and esophagus), mandibular lymph nodes, thoracic cavity, liver, adrenal glands, and possibly the kidneys. Most lesions in the organs of deglutition and thoracic cavity were compatible with gavage trauma. Moderate to severe acute/chronic inflammation and hemorrhage were observed in the larynx, esophagus, and thoracic cavity of rats of both sexes from all treatment groups. Mild to moderate hepatocellular swelling was present in 100% of the male rats treated with HC 27-S. Concurrently, 67, 100, and 83% of the treated females sacrificed following 7, 21, and 35 days, respectively, exhibited nepatocellular swelling. When graded on a severity scale of 0-4 (0 = no effect, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe), the following averages were established for hepatocellular swelling in each treatment group. (Control rats did not display this lesion and are not included.)

Time of Sacrifice	Males	Females
7 Days	2.7	1 0
21 Days	3.0	2.5
35 Days	2.8	1 2

TABLE 9.3-2. MEAN[a] ORGAN WEIGHTS (g) AND ORGAN-TO-BODY WEIGHT RATIOS[b] OF F-344 RATS FOLLOWING REPEATED ORAL ADMINISTRATION OF HALOCARBON 27-S

	7 Days		21 Days		35 Days	
			Male			
Organ	Control (6)	Test (5)	Control (6)	Test (5)	Control (6)	Test (5)
Brain	1.77 ± 0.01	1.77 ± 0.02	1.77 ± 0 02	1.79 ± 0.00	1.91 ± 0.01	1.88 ± 0.01
Ratio	0.87 ± 0.02	0.85 ± 0.01	0.80 ± 0.02	0.88 ± 0.01	0.74 ± 0.01	0.92 ± 0.03[c]
Thymus	0.23 ± 0.01	0.23 ± 0.01	0.26 ± 0 01	0 19 ± 0.01[c]	0.29 ± 0.01	0.21 ± 0.02[d]
Ratio	0.11 ± 0.01	0.11 ± 0.00	0.12 ± 0.00	0.09 ± 0 00	0 11 ± 0.01	0.10 ± 0.01
Heart	0.73 ± 0.02	0.71 ± 0.01	0.81 ± 0.03	0 72 ± 0.02	0.97 ± 0.02	0.76 ± 0.03[c]
Ratio	0.36 ± 0.01	0.34 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.37 ± 0.01	0.37 ± 0.02
Liver	6.60 ± 0.42	11.51 ± 0.33[c]	7.23 ± 0.41	17.90 ± 0.33[c]	8.08 ± 0.17	17.90 ± 0.69[c]
Ratio	3.21 ± 0.15	5.53 ± 0.12[c]	3.25 ± 0.11	8.80 ± 0.21[c]	3.12 ± 0.04	8.73 ± 0.12[c]
Kidney	1.64 ± 0.08	1.95 ± 0.04	1.78 ± 0.06	2.08 ± 0.02	2.07 ± 0.03	2.35 ± 0.10
Ratio	0.80 ± 0.03	0.94 ± 0.02[c]	0.80 ± 0.01	1.02 ± 0.01[c]	0.80 ± 0.01	1.15 ± 0.02[c]
Whole body	**204.5 ± 4.1**	**206.5 ± 2.0**	**222.2 ± 6.3**	**203.6 ± 3.1**	**259.3 ± 4.4**	**204.8 ± 6.5[c]**
			Female			
	Control (6)	Test (5)	Control (6)	Test (3)	Control (6)	Test (5)
Brain	1.64 ± 0.02	1.66 ± 0.02[d]	1.64 ± 0.01	1.71 ± 0.00[d]	1.69 ± 0.02	1.71 ± 0.02[d]
Ratio	1.20 ± 0.02	1.35 ± 0.06[d]	1.15 ± 0.03	1.19 ± 0.04	1.06 ± 0.02	1.08 ± 0.03
Thymus	0.23 ± 0.01	0.21 ± 0.03	0.23 ± 0.01	0.22 ± 0.01	0.25 ± 0.02	0.25 ± 0.02
Ratio	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0 15 ± 0 01	0.16 ± 0.01	0.16 ± 0.01
Heart	0.56 ± 0.02	0.50 ± 0.03	0.52 ± 0.01	0 50 ± 0 01	0.63 ± 0.02	0.67 ± 0.02
Ratio	0.41 ± 0.02	0.40 ± 0 02	0 37 ± 0.01	0 35 ± 0 02	0.40 ± 0.01	0.42 ± 0.01
Liver	4 04 ± 0 12	5.16 ± 0.38	4 03 ± 0 10	8 96 ± 0.12[d]	4 66 ± 0.18	7 59 ± 0.47[d]
Ratio	2.96 ± 0.05	4.14 ± 0.18[c]	2.81 ± 0 06	6.26 ± 0.29[c]	2 92 ± 0.07	4 81 ± 0.40[c]
Kidney	1.05 ± 0.03	1.11 ± 0.04	1 10 ± 0 03	1.29 ± 0.01[d]	1.26 ± 0 04	1.47 ± 0.03[d]
Ratio	0.77 ± 0.01	0.90 ± 0.03[c]	0.77 ± 0.01	0.90 ± 0.04[c]	0 79 ± 0.01	0.93 ± 0.04[c]
Whole body	**136.7 ± 2.8**	**123.7 ± 5.4**	**143.3 ± 2.5**	**143.7 ± 5.5**	**159.3 ± 3.7**	**159.0 ± 4.8**

[a] Mean ± S E M (N)
[b] Organ weight/body weight x 100
[c] Statistically different from controls at $p < 0.01$ using two factorial analysis at variance
[d] Statistically different from controls at $p < 0.05$ using two factorial analysis at variance

Minimum to mild vacuolar degeneration (diagnosed as fatty change) of adrenocortical cells was present in 100% of the treated males and 50% of the treated females killed immediately following the 21-day treatment Similarly, 67 and 83% of the males and females, respectively, killed following the 14-day recovery period also exhibited mild adrenocortical vacuolar changes. Adrenocortical changes were noted in only one control animal from the study.

Although increased weights were reported in kidneys harvested from treated rats, microscopic findings were generally unremarkable. Four of six treated male rats killed at seven days displayed mild accumulations of hyaline droplets (resorbed protein) in the cytoplasm of proximal tubular epithelial cells. Similar hyaline droplets were not recorded in males killed at the 21- or 35-day sacrifice. Similarly, relative testes weights were increased in male rats treated for 21 and 35 days.

DISCUSSION

Repeated oral dosing of HC 27-S to male and female rats resulted in unthrifty hair coat, lethargy, and diarrhea. Convulsions occurred as early as four days and continued irregularly thereafter in the female rats. All of these toxic signs are typical of acute fluoride poisoning (Haynes and Murad, 1985).

Depression of body weight gains throughout the first 21 days of the study appeared to be related to treatment and housing (metabolism cages with ground food and a daily 16-h fast period), even though the rats had a one-week acclimatization period prior to the treatment period. Following the 21-day treatment period the rats were returned to shoebox cages, pelletized food, and unfasted conditions All groups except the male test group showed a dramatic increase in mean body weight during the subsequent 14 days In addition to the weight loss as the study progressed, the rats became increasing irritable and difficult to handle, which contributed to the number of cases of gavage trauma.

The results of clinical analyses of urine samples indicated an increase in creatinine excretion during the first week of the study. Creatinine is normally excreted through the kidneys in quantities proportional to serum content (Widmann, 1973) Serum creatinine values for the test rats at the seven-day sacrifice (when urine creatinine values were elevated) indicated no significant increase above control values Nor were there significant increases in serum creatinine values at the 21- or 35-day sacrifices The significance of the increased values and whether either were indicative of kidney damage remains questionable.

The elevated concentrations of serum BUN in the male rats indicated that plasma clearance ability may have been affected Return toward normal values was not apparent following the 14-day

post-treatment period. The elevated serum albumin concentrations in both sexes of treated rats may have been related to the reduced water consumption and resultant dehydration of the test rats.

The major route of fluoride excretion is the kidneys (Haynes and Murad, 1985). In this study the daily content of inorganic fluoride in the urine of the test rats was significantly increased throughout the study. The mean concentration of inorganic fluoride in the urine of the test rats examined following 14 and 21 days of treatment ranged between 22 and 32 mg/L, approximately six to seven times that of the respective controls. Fourteen days following cessation of treatment the test rats were still excreting between 11- and 16-mg inorganic fluoride/L compared with control rats which excreted between 2 and 6 mg/L throughout the study. It is reported in Patty's Industrial Hygiene and Toxicology (1963) that the mean concentration of inorganic fluoride in the urine of Danish cryolite workers who complained of loss of appetite, shortness of breath, and nausea was 16.05 mg/L (range of 2.41 to 43.41). In those workers with less severe exposure the mean urinary concentration of fluoride was 4 81 mg/L (range of 1 78 to 11 67). It was also reported that heavily exposed aluminum workers (aluminum is produced by the electrolysis of bauxite in a bath of molten cryolite) had a mean daily urine fluoride concentration of 9.03 mg/L. In two factories in the United States, increases in the radiographic density of the bones have appeared in men whose urine was known to have contained $\geq$ 10 mg inorganic fluoride/L.

Plasma inorganic fluoride concentrations measured at 7, 21, and 35 days were not elevated when compared with controls Previous studies with CTFE oligomers (Kinkead et al., 1987) reported significantly elevated plasma fluoride seven days after a single oral dose. However, in this study the blood samples of the test animals were, at five of the six examination times, lower in fluoride concentration than the control animals It appeared as though an overcompensation occurred in the HC 27-S treated rats (possibly indicating improved renal function) resulting in abnormally lower plasma fluoride concentrations, or concentrations that peaked at less than 24 h.

The CaP ratios obtained in this study were from the mineralized matrix of rat femurs below the periosteum This area of bone should reflect any changes in bone metabolism or mineralization due to its proximity to the periosteum, the area of rapid bone turnover. The CaP ratios were in the normal physiological range of 1 3 to 2 0 (Guyton, 1976) for all but one group of male rats. The ratio greater than 2.0 (2.05 $\pm$ 0.02 SEM) from the male group treated for seven days appeared to peak at seven days, then level out over the 21 days of treatment, and then slowly decreased after cessation of treatment. Since bone turnover in rats is rapid, this was not surprising. In identically treated female rats, CaP ratios did not vary from control female CaP ratios

The activity of serum alkaline phosphatase was elevated in the treated male rats at each sacrifice period A slight increase in serum alkaline phosphatase activity in treated female rats was

observed only at the final sacrifice. The most likely explanation for the increased enzyme activity in male rats was the more severe hepatocytomegalic liver disease. Enlarged hepatocytes probably compressed canaliculi and small biliary ducts causing partial intrahepatic cholestasis. Bile flow obstruction commonly leads to the induction of alkaline phosphatase synthesis in the liver. Correspondingly, SGOT and serum glutamic pyruvic transaminase, derived from injured hepatocytes, becomes elevated in the blood. Since the rats used in the study were relatively young, their relatively high rates of bone osteoblastic activity may have contributed to the serum alkaline phosphatase level. However, the bone contribution was probably insignificant since the microscopic examination failed to disclose altered bone structure in either treated or control male rats. The slightly greater serum alkaline phosphatase activities of male controls as compared to female controls were reflective of the faster growth rate of the males compared to females. Since fluoride is incorporated into the bone by replacing hydroxyapatite with the denser fluoroapatite (calcium fluoride, Haynes and Murad, 1985), it is reasonable to expect that the CaP ratio would increase as fluoride replaces phosphorus in the bone. Fluoride is preponderantly deposited in the skeleton and teeth, and the degree of skeletal storage is related to intake and age (Haynes and Murad, 1985). This is thought to be a function of the turnover rate of skeletal components, with growing bone showing a greater fluoride deposition than bone in mature animals Although both male and female rats were identical in age during this study, male rats typically grow at a more rapid pace than females (approximately two times), which may explain the change in CaP ratio observed only in the male rats.

The description of thymus atrophy was a subjective judgment made during necropsy which was not verified by histopathologic examination. Inflammation and hemorrhage of the deglutitory organs was attributed to the overall debilitated condition of the rats which resulted in increased difficulty performing the daily gavage treatment. The gavage trauma produced chronic esophagitis which resulted in lymphocytosis and hyperplastic mesentery and mandibular lymph nodes. Hepatocellular swelling was considered to be a distinct, treatment-related finding in this study and was persistent after the 14-day post-treatment period. The cytoplasmic changes may have included proliferation of smooth endoplasmic reticulum and/or peroxisomes Studies designed to assess the ultrastructural bases for the hepatocytic lesions and to compare hepatic effects of HC 27-S with other compounds believed to cause similar hepatocytic effects are in progress It was also apparent that adrenocortical vacuolar degeneration was a treatment-related effect which remained, unreversed, following the 14-day post-treatment period

In summary, this study indicated that repeated oral administration of HC 27-S results in toxic effects not unlike those of acute fluoride poisoning. Although HC 27-S is a polymer of CTFE, the overall toxic effects were not typical of those observed following CTFE exposure Signs of nephrotoxicity, diuresis, and increased water consumption were not evident. Urine concentrating

ability was unaffected in both sexes of rat. Defluorination, indicated by increased urine fluoride content and changes in CaP ratios in the femurs of male rats, was evident throughout the study. Findings of gross liver enlargement and microscopic hepatocellular cytomegaly indicated that the liver was the primary rat organ injured by the repeated oral doses of HC 27-S used in this study.

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SECTION 10

SPECIAL CASE TOXICITY STUDIES OF NAVY MATERIALS

10.1 EVALUATION OF THE ACUTE DELAYED NEUROTOXICITY OF FOUR SHIPBOARD HYDRAULIC FLUIDS

E.R. Kinkead, S.S. Henry, B.T. Culpepper, and H.G. Wall

INTRODUCTION

Organophosphate ester-based hydraulic fluids are being used under military specifications (MIL-H-19457B and MIL-H-19457C) for fire-resistant hydraulic fluids. MIL-H-19457B specified that the material be a formulation of phosphoric acid esters and it included a requirement for administration to chickens for estimation of delayed neurotoxicity This specification has been replaced by MIL-H-19457C which requires that the material be a tri-tertiarybutylphenyl phosphate base and that triorthocresyl phosphate (TOCP) content (as estimated by chemical analysis of o-cresol) be less than 1%.

MIL-H-19457-C fluid has recently replaced MIL-H-19457-B fluid in the hydraulic systems of U.S. Navy ships. However, the possibility exists that a significant amount of residual MIL-H-19457B fluid has contaminated the replacement fluid. Hydraulic fluid samples were removed from each of two hydraulic lifts and from each of two original supply containers of the replacement fluids on a single Navy vessel. These samples were evaluated for o-cresol by the method described in military specification MIL-H-19457C The in-house chemical analyses indicated that the fluids from the two hydraulic lifts contained concentrations of o-cresol 5 to 10 times greater than the material from one of the original containers and gave gas chromatographic (GC) patterns similar to phosphate triester materials The GC patterns suggested that the fluids may be a mixture of several hydraulic fluids (Leahy et al., 1988) An acute delayed neurotoxicity study was performed on each of the four samples provided by the Navy Medical Research Institute/Toxicology Detachment (NMRI/TD) to determine if contamination with MIL-H-19457B fluid has created a potential health hazard.

Many organophosphate ester compounds have been found to cause delayed neurotoxic effects in man (Doull et al , 1979) A single exposure to certain neurotoxic organophosphate ester compounds has been reported to produce axonal damage after a delay of 8 to 10 days. Low-level nerve injury may occur in humans after chronic exposure to these compounds. Similar neurotoxic effects have been demonstrated in adult chickens and cats after exposure to TOCP (Beresford and Glees, 1963). This study was designed to determine if delayed neurotoxic effects could result from exposure to the sample fluids removed from a U S Navy ship

MATERIALS AND METHODS

Animals

Delayed neurotoxicity potential was evaluated using debeaked female leghorn hens (*Gallus domestica*, Carey Nick 320 hybrid, Carey Farms, Inc., Marion, OH), eight to 14 months of age, weighing between 1 4 and 2 3 kg The hens were identified by leg bands and group housed in 3 ft. x 6 ft. pens to allow free movement. Food (MannaPro, Eggmaker 15 Crumbles) and water were provided *ad libitum*. Hens were maintained on a 12-h light/dark cycle

The supplier provided additional flock history and information on husbandry practices. Table 10 1-1 lists the vaccinations administered to the flock No pesticides were ever applied to hens used in this study, and no disinfectants were used while birds were in the poultry houses. When poultry houses become vacant, they are cleaned and disinfected with formaldehyde. The supplier indicated that the flock had not experienced any disease problems.

TABLE 10.1-1. FLOCK VACCINATION HISTORY[a]

Vaccination	Age of Hen
Marek's Disease	1 day
Infectious bronchitis	2 weeks
Infectious bursal disease	2 weeks
Newcastle disease	2 weeks
Infectious bronchitis (booster)	10 - 12 weeks
Newcastle disease (booster)	10 - 12 weeks
Fowlpox	20 - 24 weeks

[a] Provided by Carey Farms, Inc , Marion, OH

Test Materials

The four hydraulic fluid samples, supplied by NMRI/TD, are listed in Table 10.1-2.

TABLE 10.1-2. HYDRAULIC FLUID SAMPLES.

Sample ID	Source
907-01	FMC Corporation (original container)
907-02[a]	Stouffer Chemical Company (original container)
907-03	AFT-HPU, Sliding block and component, lift no 1
907-04	FWD-HPU, Sliding block and component, lift no 2

[a] This material contained two discrete layers as received The upper layer was designated 907-2A The lower (aqueous) layer was designated 907-2B The fluid was mixed thoroughly prior to dosing to ensure that the hens received representative amounts of both layers

The total o-cresol content of each of the samples was measured following an extraction with ethyl ether and GC analysis Analysis of the shipboard materials indicated that the materials from the

lifts, designated 907-03 and 907-04, contained o-cresol, as did the material provided in the drum sample designated 907-01 Materials from the layers in the second drum sample, designated 907-02A and 907-02B, did not contain o-cresol The three samples containing o-cresol gave characteristic GC patterns consistent with those obtained from reference phosphate triester materials (Leahy et al , 1988).

Table 10 1-3 provides the concentrations of o-cresol expressed as percent TOCP from the following MILSPEC calculation.

$$\text{weight \% o-cresol} = \frac{(\text{weight standard}) \times (\text{area sample [GC]}) \times 100}{(\text{area standard [GC]}) \times (\text{weight sample})}$$

TOCP, practical grade, lot #B9A, was obtained from Eastman Kodak Co. for use as a positive control. Commercial grade corn oil (Mazola) was used to dilute the hydraulic fluids and also served as a negative control. The corn oil was tested for peroxide content prior to use.

TABLE 10.1-3. PERCENT TOCP CONTENT OF ANALYZED HYDRAULIC FLUIDS[a]

Sample	% TOCP
907-01	0.006
907-02A	-------a
907-02B	-------a
907-03	0 031
907-04	0.063

[a] o-Cresol not detected in sample

ACUTE DELAYED NEUROTOXICITY

The design of this study followed the requirements of military specification MIL-H-19457B All hens were weighed prior to the start of the study and weekly thereafter Test substances were administered to unfasted adult hens by oral intubation employing a 3-cc syringe fitted with a 15-cm infant feeding catheter Doses were administered on five consecutive days beginning on Monday The hydraulic fluids and TOCP were diluted in corn oil to the appropriate dose Each hen was weighed prior to the initial dose and 1.0 mL/kg body weight was administered by gavage The dosing regimen was as follows

Hydraulic fluid samples:	Groups of four hens each were treated with 420, 360, 300, or 240 mg/kg/day for five days
TOCP positive controls:	Groups of four hens each were treated with 90, 75, or 60 mg/kg/day for five days
Corn oil:	Twelve hens were given the maximum total volume of fluid equal to that given test animals (i e , 1 0 mL/kg)

Observations and grading began seven days after the first dose and continued three times a week (Monday, Wednesday, and Friday) until 30 days after the initial dose. The following scoring system was used.

Symptom-free	0 points
Doubtful or minor symptoms	2 points
Positive paralytic symptoms	8 points
Advanced paralytic symptoms	12 points
Death	16 points

During observation and grading, the chickens were removed from their enclosures and placed on a rubber mat to provide sure footing Symptoms observed in test hens during the observation period were compared with those seen in the TOCP-treated hens. Scores reported represent an average of the scores of the three observers

All test and control hens were examined for gross pathology at death. All surviving chickens were sacrificed upon completion of the observation period. The entire brain, spinal cord, and both sciatic nerves (with attached gastrocnemius muscles) were collected for histopathologic examination. Histologic sections were prepared of the medulla, cerebellum, optic lobes, and frontal cortex of the brain; cervical, thoracic, and lumbosacral segments of the spinal cord; the proximal, middle, and distal thirds of one sciatic nerve, the entire gastrocnemius, and any observed gross lesions Duplicate sciatic nerve and spinal cord sections from at least three hens per treatment group were stained with Bodian's Stain to demonstrate cytoplasm in neuron cell bodies and processes, and luxol fast blue to demonstrate myelin.

The following criteria were used for determining the three significant light microscopic neural diagnoses applied in this study

1. **Axoplasmic degeneration** - Focal swelling of the axis cylinders with increased staining of axoplasm or discontinuity in the axoplasm without detectable effect on adjacent myelin of the affected nerve fiber

2. **Demyelination** - Focal discontinuity in myelin without detectable damage to adjacent axoplasm in the affected nerve fiber Furthermore, the damaged myelin should appear as a condensed focus, isolated by a clear halo from unaffected myelin (which has a reticular pattern)

3 **Nerve fiber degeneration** - Simultaneous axoplasmic degeneration and demyelination involving the same segment of a nerve fiber, or discontinuity in a nerve fiber that is accompanied by reactive inflammation or a reparatory response

EXPERIMENTAI RESULTS

Neurotoxic signs were observed in 11 of the 12 hens that received TOCP, one hen in the lowest TOCP dosage group (60 mg/kg) exhibited only doubtful or minor symptoms The four test groups and the corn oil control showed no signs of neurotoxicity Two of the test chickens died during the observation period, however, neither animal showed neurotoxic symptoms prior to death.

The incidence of gross lesions was very low and no statistically significant group differences in gross lesions were revealed Gross lesions that were histologically confirmed included hepatic fibrinous serositis, necrotizing hepatitis, hepatic hemangioma (hemangioendothelioma), chronic portal hepatitis, chronic oviduct inflammation, chronic ovary inflammation, chronic vegetative endocarditis, and chronic vegetative myocarditis

The spinal cords and sciatic nerves were the only organs with statistically significant group differences in the incidence and severity of histologic lesions (Tables 10.1-4 and 10 1-5). Although several neural lesions occurred in each treatment group, severe lesions occurred most frequently in hens exposed to TOCP (positive controls) Gliosis and lymphocytic perivascular cuffing was seen in brain sections from hens in each dose group; however, group differences were not significant

TABLE 10.1-4. INCIDENCE OF STATISTICALLY SIGNIFICANT HISTOLOGIC LESIONS

Group Animals Per Group	Corn oil 12	01 16	02 16	03 16	04 16	TOCP 12
No. animals with lesions						
Axoplasmic degeneration, spinal cord	1	5[a]	1[b]	0[b]	1[b]	9[c]
Nerve fiber degeneration, spinal cord	2	3[b]	5[a]	10[d]	7[a]	10[c]
Nerve fiber degeneration, sciatic nerve	0	4[a]	6[a]	3[a]	2[a]	12[c]

[a] Statistically different from TOCP, $p < 0.05$ using one factorial analysis of variance corrected for multiple comparisons
[b] Statistically different from TOCP, $p < 0.01$ using one factorial analysis of variance corrected for multiple comparisons
[c] Statistically different from corn oil, $p < 0.01$ using one factorial analysis of variance corrected for multiple comparisons
[d] Statistically different from corn oil, $p < 0.05$ using one factorial analysis of variance corrected for multiple comparisons

Non-neural histologic lesions were limited primarily to a 33% or less incidence of chronic interstitial myositis, a 13% or less incidence of myofiber regeneration, and those histologic diagnoses associated with the observed gross lesions The non-neural histologic lesions were observed in both test and control hens.

SUMMARY/DISCUSSION

Past studies (e g., Abou-Donia, 1981) have shown the adult female chicken to be the preferred laboratory animal for evaluating the delayed neurotoxic potential of organophosphate esters In order to maintain equilibrium and locomotion, the chicken, a biped, must have well-developed neuromuscular control. Surviving hens, receiving five consecutive oral doses of up to 420-mg test

TABLE 10.1-5. STATISTICALLY SIGNIFICANT LESION SEVERITY MEANS[a]

	Group					
Animals Per Group	Corn oil 12	01 16	02 16	03 16	04 16	TOCP 12
Diagnosis						
Axoplasmic degeneration, spinal cord	083	313[b]	067[b]	000[b]	063[b]	1 417
Nerve fiber degeneration, spinal cord	167	188[b]	333[b]	812[b]	438[b]	2.333
Gliosis, spinal cord	000	438	200	250	375	917
Demyelination, sciatic nerve	750	750[b]	1 113[b]	1 188[b]	1 063[b]	2.333
Nerve fiber degeneration, sciatic nerve	000	313[b]	400[b]	.188[b]	.125[b]	3.167
Schwann cell hyperplasia, sciatic nerve	.000	.250	.133[c]	.063	.063[b]	.833

[a] Scoring system defined as 0 = no lesion, 1 = minor or very slight, 2 = slight, 3 = moderate, 4 = marked, 5 = severe Group scores are calculated by dividing the sum of individual scores by the number of affected hens
[b] Statistically different from TOCP, $p < 0.01$ using one factorial analysis of variance corrected for multiple comparisons
[c] Statistically different from TOCP, $p < 0.05$ using one factorial analysis of variance corrected for multiple comparisons

material/kg body weight, remained neurologically asymptomatic throughout a 30-day observation period

Neural lesions having a frequency and severity consistent with organophosphate ester-induced delayed neuropathy (OPIDN) were limited to the TOCP positive controls. None of the four tested hydraulic fluids caused lesions consistent with OPIDN.

Evaluations of the incidence and severity of background neural lesions are crucial to any neurohistopathologic assessments of chemically induced neurotoxicity. In this study, demyelination was seen in most hens, regardless of their treatment group, however, where seen, the lesions were limited to isolated foci, usually single node areas within nerve fibers Axoplasmic degeneration also was seen which involved single node segments Nerve fiber degeneration simultaneously involving myelin and axons was rarely seen in hens other than the TOCP positive controls. These lesions, as well as gliosis and lymphocytic inflammation, are common background findings in old hens (Bickford and Sprague, 1982) The more extensive lymphocytic inflammation seen in some hens resembled neural lesions associated with Marek's Disease, a disease sometimes appearing in flocks that have been vaccinated Neural lesions having severity consistent with those of OPIDN (Bickford and Sprague, 1982, Olajos et al , 1986) were found only in the TOCP-dosed hens Severity rather than incidence is the most important criteria for assessing delayed neurotoxicity in hens with neural background lesions

The gross lesions that were confirmed histologically are considered to be age-related or infection-related diseases having a very low incidence Their presence would not be expected to

influence the action of the chemicals administered to hens being evaluated Under the conditions of this study, none of the four hydraulic fluids tested were neurotoxic

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Leahy, H.L., H.C. Higman, and E.R. Kinkead. 1988. Subchronic Studies of Shipboard Hydraulic Fluids and Bioassay. In: W.E. Houston and R.S. Kutzman, eds. *1987 Toxic Hazards Research Unit Annual Report.* AAMRL-TR-88-008, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-88-11, Bethesda, MD: Naval Medical Research Institute

Olajos, E.J., J.D. Bergmann, and H.G. Wall. 1986. O-Ethyl-o'-diisopropyl-aminoethyl methyl-phosphonite (QL) neurotoxicity in hens, CRDC-TR-84129. ATTN: SMCCR-SPD-R. Aberdeen Proving Ground, MA: U.S. Army Chemical Research and Development Center. Available from Commander, U.S. Army Chemical Research and Development Center

APPENDIX A

TOXIC HAZARDS RESEARCH UNIT PERSONNEL LIST

TOXIC HAZARDS RESEARCH UNIT ORGANIZATIONAL CHART

OFFICE OF DIRECTOR

Raymond S. Kutzman, Ph.D.
Rory B. Conolly, Sc.D.

Staff:
Gaston, Daphne L.
Stokes, James S.

ADMINISTRATION

Lois A. Doncaster
Supervisor

Staff:
Angell, Mary Ann
Kinney, Lowell E.
Roth, Judith M.
Smith, Danita H.

BIOMETRY

Carlyle D. Flemming
Senior Statistician

Staff:
Smith, Kimberly B.

QUALITY ASSURANCE

Mathias G. Schneider, Jr.
Coordinator

Staff:
Godfrey, Susan M.

SAFETY

Larry R. Snelling
Health and Safety Representative

TOXICOLOGY

Henry G. Wall, D.V.M., Ph.D.
Manager

Staff:
Bailey, Therlo C., Sr.
Barwick, Darwynn L.
Bennett, Lisa A.
Bunger, Susan K.
Calderon, Mark A
Deiser, Patricia A
DelRaso, Nicholas J.
Dille, Susan E.
Drerup, Joanne M
Godfrey, Richard J.
Godin, C Steven, Ph D.
Helton, C Douglas
Henson, Bill R.
James, Clarence
Kinkead, Edwin R.
Malcomb, Willie J.
Miller, Karen S.
Neely, Gloria A.
Nicholson, Jerry W.
Perkins, Rodney A.
Smith, Jessie L.
Wagner, Sharon L.
Wilson, Janet L.

ENGINEERING & CHEMISTRY

Robert L. Carpenter, Ph.D.
Manager

Staff:
Auten, Kenneth L.
Brade, Donald W., P.E.
Brewer, John A.
Clendenin, Timothy L.
Doarn, Charles R.
Doty, Deirdre A.
Higman, Howard C.
Kimmel, Edgar C., Ph.D
Leahy, Harold F.
Pollard, Daniel L.
Sayers, William R.
Smutak, Donald A.
Soloman, Kenneth G.
Sonntag, William B
Yerkes, Kirk L

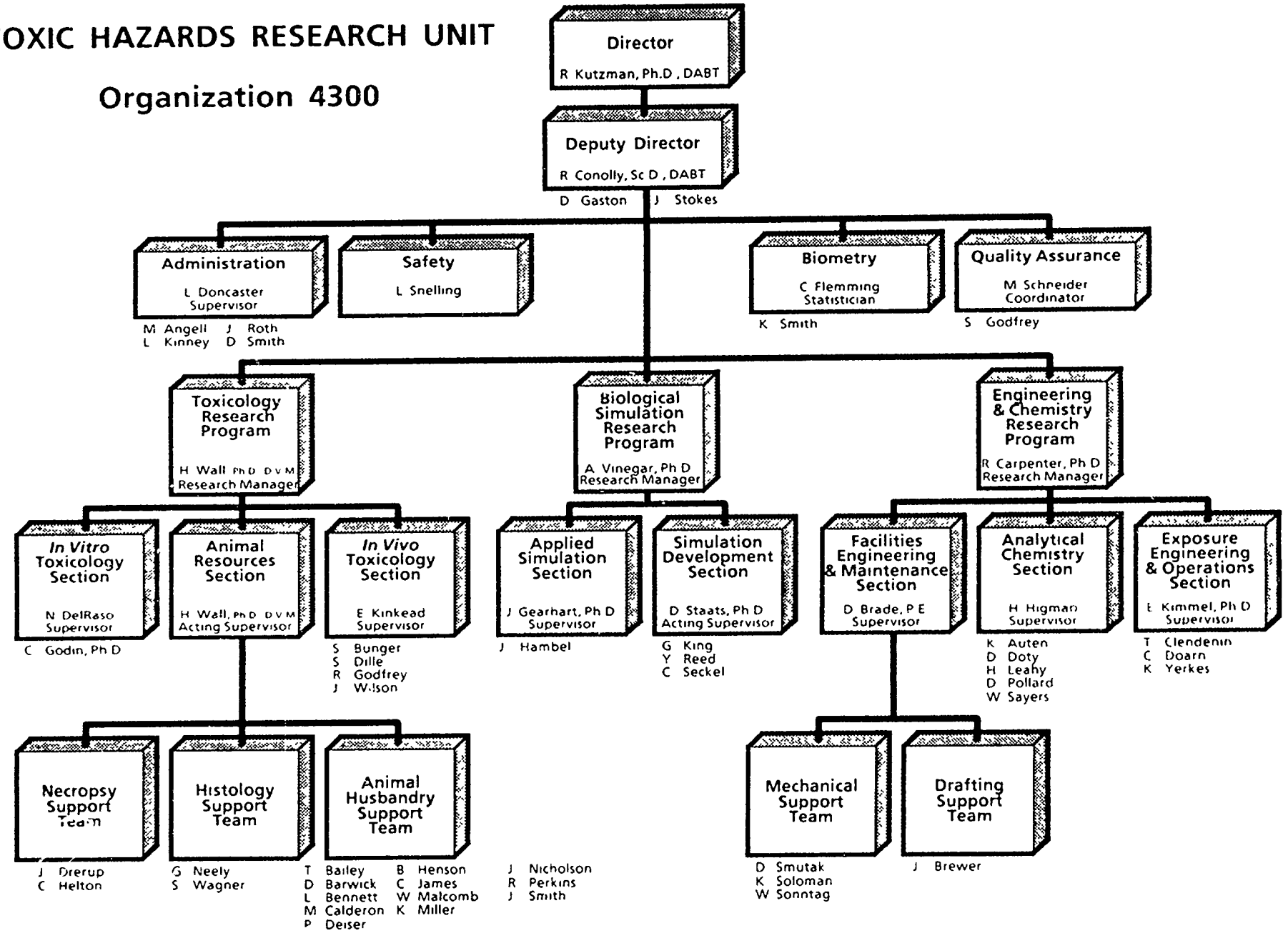
BIOLOGICAL SIMULATION

Allen Vinegar, Ph.D
Manager

Staff:
Gearhart, Jeffery M., Ph D.
Hambel, Jane M.
King, Greg A.
Reed, Yvonne M.
Seckel, Constance S.
Staats, Dee Ann, Ph.D

TOXIC HAZARDS RESEARCH UNIT

Organization 4300



APPENDIX B

QUALITY ASSURANCE

This report covers Toxic Hazards Research Unit (THRU) Quality Assurance (QA) activities from October 1987 through September 1988.

The QA Coordinator completed the Quality Assurance Program Plan for the THRU This plan describes the way THRU studies are conducted to produce reliable, quality data. The plan was reviewed and approved by THRU management before it was submitted to the NSI Technology Services Corporation – Environmental Sciences (NSI-ES) QA Committee for additional review and approval. The QA Program Plan has now been implemented and incorporated into THRU research programs.

The QA Unit participated in the activities of the NSI-ES QA Committee. Those activities that impacted the THRU were reviewed and revised to meet THRU needs. Training courses in QA and Good Laboratory Practices (GLPs) were developed A course in Basic Quality Assurance was presented to THRU, Air Force, and Navy laboratory personnel. The course was determined to be a success by the participants.

The QA Unit completed several activities related to study records. A vital technical records inventory of archived and current study records was completed at the request of NSI-ES management. At the request of the Air Force, historical study data were gathered for reporting to the World Health Organization. A continuing process was the microfilm backup of study records. The filming of archived records was completed, and filming of records from recently completed studies was initiated.

The QA Coordinator participated in several meetings and conferences where the future direction of GLP regulations was discussed with representatives from regulatory agencies (Food and Drug Administration and Environmental Protection Agency) and regulated companies and laboratories. Knowledge gained from these discussions has been used to update current THRU application of GLPs and to develop procedures that will anticipate future changes and ensure the quality of the work produced from the THRU.

The THRU QA Unit conducted systems audits for two programs Procedures, organization, and personnel for the THRU *In Vitro* Toxicology Section were reviewed Recommendations were made that would further GLP compliance The QA Unit was permitted to review the Air Force Clinical Chemistry program to identify aspects that might produce data that were not in compliance with THRU quality requirements. None were identified

The THRU QA Unit completed study report and data audits for the listed Study Requests during the previous year.

STUDY REQUEST NUMBER	STUDY TITLE
TH 0-10.1	Subchronic Studies on Chlorotrifluoroethylene
TH 0-10.2	Dermal Studies of Air Force Clothing Samples
TH 0-20.4	Dermal Absorption Kinetics of Selected IRP Chemicals
TH 0-20.5	Acute Toxicity Testing of IRP Chemicals
USN 0-60.2	Dermal Toxicity Testing of Activated Peroxide
USN 0-70.2	Evaluation of the Subchronic Toxicity of Orally Administered Halocarbon 27-S

The QA Unit conducted procedure and data audits during the course of the following studies.

STUDY REQUEST NUMBER	TEST PHASE	AUDIT DATES
TH 0-10.1	Skin Sensitization	28-30 Oct 1987 11-12 Nov 1987
	90-Day Inhalation	18 Nov 1987
	Biological Simulation Specimens	8-10, 14, 23, 28, 31 Dec 1987 27 Jan 1988
TH 0-10.2	Animal Receipt	10 Nov 1987
	Skin Sensitization	6, 8 Jan 1988
TH 0-10.3	Primary Hepatocyte Assay	9 Aug 1988
TH 0-20.3	Trichloroethylene Metabolite Assay	10 Aug 1988
TH 0-20.4	Dermal Absorption Data	25 Aug 1988
TH 0-20.6	Perchloroethylene Data	10 Aug 1988
	Kidney Perfusion	5 May 1988

STUDY REQUEST NUMBER	TEST PHASE	AUDIT DATES
TH 0-30.9	Interim Data Audit	23 Aug 1988
TH 0-40.2	Interim Data Audit	11 Aug 1988
TH 0-40 4	Liver Hepatectomy Initiation Promotion	23 Aug 1988 14 Sep 1988 27 Sep 1988
TH 0-50.4	Partition Coefficient Data	11 Aug 1988
TH 0-51.4	Target Organ Toxicity	26-28 Jul 1988 2, 3, 9, 10, 16-18 Aug 1988
	PB-PK Specimen Collection	28, 29 Jul 1988 1, 2, 4, 8, 9, 12, 13, 15, 18, 19, 25 Aug 1989
	Mouse Genotoxicity	13-15, 22, 29 Jul 1988 3, 4 Aug 1988
	Clinical Chemistry Data	8 Sep 1988
USN 0-80 5	Neurotoxicity	4 Jan 1988 1, 8 Feb 1988

APPENDIX C

SAFETY AND HEALTH PROGRAMS

The Toxic Hazards Research Unit (THRU) is developing a Safety Procedures Manual specific to its activities to supplement the NSI Technology Services Corporation – Environmental Sciences (NSI-ES) Health and Safety Manual This manual will represent and clarify actual safety procedures followed at this facility The following sections have been written and are being implemented.

Hazard Communication Training

This program was designed to train employees in the safe handling of hazardous substances. NSI-ES requires that personnel are provided with manufacturer's safety information when working with hazardous materials in their laboratories The Hazard Communication Program requires that the following information is available to and maintained by THRU personnel. (1) chemical container labels meeting set labeling requirements; (2) material safety data sheets (MSDS's); and (3) safe handling procedures. Training classes will be provided to new employees joining the THRU.

Chemical Accountability Procedures

A general policy for creating and maintaining a functional chemical inventory has been developed The chemical inventory will serve as a tool to assist THRU researchers with the purchase, accounting, safe storage, and disposal of all chemicals in use at the facility.

Medical Surveillance

Additional medical services were required for the management of potential non-human primate bites and scratches that could lead to Herpes B Virus infections A search was instituted for a medical facility to provide the comprehensive medical support that was needed.

Facility Safety Inspections

A detailed inventory of all safety equipment and its location was established This list will be used to facilitate future safety equipment inspections and to follow up on needed equipment repairs.

An Armstrong Aerospace Medical Research Laboratory Annual Facilities Safety Inspection gave this facility an "excellent" rating. Few items were noted in the results of the evaluation.

APPENDIX D

ANIMAL HUSBANDRY TECHNICIAN TRAINING PROGRAM

Toxic Hazards Research Unit (THRU) Animal Husbandry Support Team technicians are responsible for the care of all animals housed in Buildings 79, 429, 433, 838, and 839 in Area B, Wright-Patterson Air Force Base. Responsibilities include animal care during quarantine and holding, as well as during the pre- and postexposure periods of the in-life phase of scientific studies. Diversified training of the animal husbandry technicians permits additional animal handling and laboratory support capability to approved research protocols.

The numbers of THRU animal technicians currently certified by the American Association for Laboratory Animal Science (AALAS) are listed below.

Laboratory Animal Technologists	–	3
Laboratory Animal Technicians	–	3
Assistant Laboratory Animal Technicians	–	1

During this year, one technician was certified as a Laboratory Animal Technician and one technician was certified as an Assistant Laboratory Animal Technician. The outline of the AALAS course was described in detail in a previous THRU annual report (MacEwen and Vernot, 1975). All references listed by AALAS that are utilized in preparing for examinations are available through the THRU and U S. Air Force libraries.

To increase the efficiency of new technicians, a special training program has been planned that will enable new technicians to rapidly acquire proficiency in basic animal technology and animal care routines. This program includes self-study exercises, mandatory reading of animal husbandry standard operating procedures, seminars, demonstrations, on-the-job training, and a formal evaluation of animal husbandry knowledge and skills after 60 days of employment.

Ongoing training and education for animal husbandry technicians during the past year included

- Purina Laboratory Animal Care Course, completed by four new technicians;
- in-life computerized animal data acquisition using the Xybion PATH/TOX System, 12 technicians;
- nonhuman primate quarantine procedures, three technicians;
- basic quality assurance, three technicians;

- nonhuman primate surgery and anesthesia; three technicians;
- AALAS Certification training sessions, three technicians;
- local training sessions for all technicians on the following subjects:
  - restraint of livestock;
  - safety: "Think Snow" film and asbestos monitoring during sandblasting in Building 839;
  - cage washing, detergents, and temperatures;
  - personal hygiene;
  - prevention and management of non-human primate bites and scratches;
  - diseases and restraint of the cat;
  - effects of selected environmental factors on animal health and research results;
  - latest developments: Herpes B in monkeys/man;
  - materials safety training;
  - correct and legal housing for laboratory animals;
  - the use of physiologically-based pharmacokinetic models in toxicological research; and
  - operation and maintenance of the tunnel and automatic cage washers.

A complete list of the programs and training materials used by the animal husbandry technicians is provided below. The numbers in parentheses indicate the percentage of technicians in the current group that have completed these courses

Purina Animal Care Course - self-study program	(75)
Biotech Small Animal Series - small animal bleeding, oral dosing, handling, and restraint of laboratory animals	(50)
Good Laboratory Practices	(67)
Practical Training List	(33)
Advanced Practical Training List	(33)
Toxic Hazards Research Unit Animal Care	(50)
Laboratory Animal Medicine and Science Autotutorial Series	(50)
Dome Flight Training	(67)

Stark/McBride Technologist Correspondence Course	(42)
Medical Terminology	(25)
Toxic Hazards Research Unit Parapathologist Video Tapes	(58)
Laboratory Animal Technologist Video Tapes - preparation for AALAS certification	(17)

A cross-training program that was initiated in 1987 was implemented throughout the past year. Technicians were rotated among a variety of animal care and research support assignments on a monthly schedule to increase their capabilities, value, and versatility.

REFERENCE

MacEwen, J.D. and E.H. Vernot. 1975. Toxic Hazards Research Unit Annual Technical Report. Report No. AMRL TR-75-57, Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

APPENDIX E

SUBMITTED TECHNICAL REPORTS, LETTER REPORTS, AND JOURNAL PUBLICATIONS

Technical Reports

Kinkead, E.R., B.T. Culpepper, S.S. Henry, D.L. Pollard, E.C. Kimmel, V.L. Harris, R.S. Kutzman, M. Porvaznik, and R.H. Bruner. 1987. Evaluation of the Acute Toxicity of Four Water-in-Oil Emulsion Hydraulic Fluids. Report No. AAMRL-TR-87-063, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory NMRI-87-51, Bethesda, MD: Naval Medical Research Institute. November.

Gardner, D.E., W.E. Houston, and **R.S. Kutzman.** 1987. Proceedings of the 16th Conference on Toxicology, 28, 29, and 30 October 1986. Report No. AAMRL-TR-87-065, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. NMRI-87-68, Bethesda, MD: Naval Medical Research Institute December

Houston, W.E. and **R.S. Kutzman.** 1988 1987 Toxic Hazards Research Unit Annual Report Report No. AAMRL-TR-88-008, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-88-11, Bethesda, MD: Naval Medical Research Institute. March.

Steele, V.E., B.P. Wilkinson, J.T. Arnold, and **R.S. Kutzman.** 1988 The Study of Beryllium Oxide Genotoxicity in Cultured Respiratory Epithelial Cells Report No AAMRL-TR-88-023, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. May.

Letter Reports

Kinkead, E.R. and **V.L. Harris.** 1987 Evaluation of the Acute Inhalation Toxicity of Bel-Ray Syncom 1400 - Water Solution August.

Kinkead, E.R., B.T. Culpepper, and **S.S. Henry.** 1987 Determination of the Acute Skin Irritation of an "Activated Peroxide" Decontaminant Using Varied Exposure Regimens. December

Kinkead, E.R., S.S. Henry, B.T. Culpepper, R.S. Kutzman, and C J Hixson. 1988 The Toxicity of Installation Restoration Program (IRP) Leachate Samples Following Single and Repeated Treatment February.

Kinkead, E.R., S.S. Henry, B.T. Culpepper, and **R.S. Kutzman.** 1988. The Evaluation of the Sensitization and Acute Dermal Irritation Potential of Air force Candidate and Inventory Clothing Materials. March

Serve, P. 1988. A Study of the Metabolism and Nephrotoxicity of Methylcyclohexane, Isopropylcyclohexane, and Tertbutylcyclohexane in Male Fischer 344 Rats March

Vinegar, A. and **D. Brown.** 1988 Development of an Isolated Ventilated Perfused Lung Preparation April.

Pollard, D.L. 1988 Dermal Exposure to Hydrazine Vapors April

Names which are bolded represent NSI-ES employees

Journal Publications

Conolly, R.B., R.E. Reitz, H.J. Clewell, III, and M.E Andersen. 1988. Biologically structured models and computer simulation: Application to chemical carcinogenesis. *Comments in Toxicology* 2:305-319.

Conolly, R.B., R.E. Reitz, H.J. Clewell, III, and M.E. Andersen 1988. Pharmacokinetics, biochemical mechanism and mutation accumulation: A comprehensive model of chemical carcinogenesis. *Tox. Letters* 43: 189-200.

Steele, V.E., B.P. Wilkinson, J.T. Arnold, and **R.S. Kutzman.** In Press. The study of beryllium oxide genotoxicity in cultured respiratory epithelial cells. *Inhal. Toxicol.*

Journal Publications by NSI Employees (work not conducted at THRU)

Culpepper, B.T., A.J. Sheratt, and W C. Lubawy. 1988 Prostacyclin and thromboxane formation following chronic exposure to cigarette smoke condensate administered via osmotic pumps in rats. *J. Pharmacol Method* 20: 47-56.

Culpepper, B.T., A.J. Sheratt, and W.C. Lubawy. 1988. Relative participation of the gas phase and total particulate matter in the imbalance of prostacyclin and thromboxane formation seen after chronic cigarette smoke exposure. *Prostaglandins, Leukotrienes, and Essential Fatty Acids.* 34:15-18.

Godin, C.S. and P. Crooks. 1988. *N*-methylation of nicotine enantiomers by human liver cytosol. *Biochem. Pharmacol.* 40:153-154.

Godin, C.S., P. Crooks, S. Ansher, and W. Jakoby. 1988. Substrate specificity in the *N*-methylation of azaheterocycles by rabbit liver amine *N*-methyltransferases. *Biochem. Pharmacol.* 37:1673-1677.

Godin, C.S., P. Crooks, and A. Houdi. 1988 The effect of continuous nicotine administration on urinary histamine and *N-methylhistamine* levels in the guinea pig *Toxicol. Lett.* 44:161-166.

Godin, C.S., C Gairola, A. Houdi, and P. Crooks. 1988 Stereospecific *N*-methylation of nicotine by pulmonary alveolar macrophages from guinea pigs. *J. Pharm. Pharmacol.* 40:724-726.

Godin, C.S., C. Nwosu, A. Houdi, L. Damani, and P Crooks. 1988. Enantioselective metabolism during continuous administration of S-(-)- and R-(+)-nicotine isomers in guinea pigs. *J. Pharm. Pharmacol.* 40:862-869.

Mattie, D.R., M.J. Parnell, **R.L. Carpenter, R. S. Kutzman,** and **V.E. Steele.** 1988. Evaluating the toxicity of beryllium oxide-containing rocket motor exhaust particles. Proceedings of the 1988 JANNAF Safety and Environmental Protection Subcommitt Meeting, pp 297 - 303. CPIA Publication 485 Chemical Propulsion Information Agency, Laurel, MD 20707

Names which are bolded represent NSI-ES employees

APPENDIX F

PRESENTATIONS AT SCIENTIFIC MEETINGS

A Model for Respiratory Tract Deposition, Retention and Clearance of Particles, Written in ACSL. 1987. Vinegar, A., E C. Kimmel, and R B. Conolly NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431 Presented at the Symposium on Extrapolation Modeling of Inhaled Particles and Gases: Lung Dosimetry at Duke University Medical Center, Durham, NC, 8-10 October.

A Model for Respiratory Tract Deposition, Retention and Clearance of Particles, Written in ACSL. 1987. Vinegar, A., E C Kimmel, and R B Conolly. NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431 Presented at the Southeastern Simulation Conference, Huntsville, AL, 19-21 October

Quantitative Modeling of Cytotoxicity, Tissue Replication, and Mutation Accumulation. 1987. Conolly, R B., NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431; Reitz, R.H , Dow Chemical, Midland, MI 48674; and Clewell, H J., AAMRL/TH, Wright-Patterson Air Force Base, OH 45433. Presented at the 17th Conference on Toxicology, Holiday Inn Conference Center, Fairborn, OH, 3-5 November

Modeling and Measurement of Expired Chloropentafluorobenzene (CPFB) After an Inhalation Exposure. 1987. Vinegar, A. and D.W. Winsett, NSI Technology Services Corp., 101 Woodman Drive, Dayton, OH 45431. Presented at the 17th Conference on Toxicology, Holiday Inn Conference Center, Fairborn, OH, 3-5 November.

An Inexpensive Rat Restraint for Collection of Blood Samples. 1987. Blackford, R K., J L Smith, R.M. Ruppert, T.C. Bailey, and W.J Malcomb, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431. Presented at the National Meeting of the American Association of Laboratory Animal Sciences, Denver, CO, 8-12 November

Developing a Non-Irritating Guinea Pig Wrap for Sensitization Studies. 1987 Dille, S E and S.S. Henry, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431. Presented at the National Meeting of the American Association of Laboratory Animal Sciences, Denver, CO, 8-12 November

Biologically-Based Computer Simulation of Dose-Response (DR) Curves for Cytotoxic Chemical Carcinogens. 1988 Conolly, R B , NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431; Clewell, H.J., AAMRL/TH, Wright-Patterson Air Force Base, OH 45433; Reitz, R H , Dow Chemical, Midland, MI 48674, and Andersen, M E , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433. Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February

A Biologically-Based Computer Simulation Model for Hepatotoxicity. 1988 Gearhart, J M , and L J Goodpaster, NSI Technology Services Corp., 101 Woodman Drive, Dayton, OH 45431, Andersen, M E , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433, and R B Conolly, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431 Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February

Dermal Absorption Kinetics of Neat and Aqueous Volatile Organic Chemicals. 1988 Morgan, D L , J.R Tuschall, and R S. Kutzman, NSI Technology Services Corp , RTP, NC & Dayton, OH; and Mattie, D R , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433 Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February

Physiologically-Based Computer Simulation of Chloropentafluorobenzene (CPFB) Pharmacokinetics and Its Quantitation in Expired Breath: A Non-Invasive Tool for Evaluating Exposure History. 1988 Vinegar, A , D W Winsett, and R.B Conolly, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431; and Andersen, M.E., AAMRL/TH, Wright-Patterson Air Force Base, OH 45433. Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February

***In Vitro* LC_{50} Determination of Solubilized 2,3,4-Trimethylpentane Using Primary Rat Hepatocytes.** 1988 DelRaso, N J., NSI Technology Services Corp., 101 Woodman Drive, Dayton, OH 45431, and Mattie, D.R , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433. Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February.

Determination of the Subchronic Oral Toxicity of Halocarbon 27-S. 1988 Kinkead, E R., B T Culpepper, S S Henry, and R.S Kutzman, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431; Wyman, J F , NMRI/TD, Wright-Patterson Air Force Base, OH 45433; and Bruner, R H , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433 Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February.

Computer Simulation of Expired Chloropentafluorobenzene (CPFB) After Inhalation Exposure. 1988. Vinegar, A , D W Winsett, and R B Conolly, NSI Technology Services Corp., 101 Woodman Drive, Dayton, OH 45431; and Andersen, M E , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433 Presented at the 1988 Annual Meeting of Society of Toxicology, Loews Anatole Hotel, Dallas, TX, 16-19 February

Dermal Absorption Kinetics of Neat and Aqueous Volatile Organic Chemicals. 1988 Kutzman, R.S , NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431 Presented at the Symposium on Dermal Toxicity, Raleigh, NC, 1 March

Evaluating the Toxicity of Beryllium Oxide-Containing Rocket Motor Exhaust Particles. 1988 Mattie, D R and M J Parnell, AAMRL/TH, Wright-Patterson Air Force Base, OH 45433; Carpenter, R.L and R S Kutzman, NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431; and V E Steele, NSI Technology Services Corp , Research Triangle Park, NC 27709 Presented at the 1988 JANNAF Safety and Environmental Protection Subcommittee Meeting, Naval Postgraduate School, Monterey, CA, 23-27 May.

Calcium/Phosphorus Ratios of Femurs of Male and Female Fischer 344 Rats Exposed to Halocarbon 27-S. 1988 Mattie, D R and J J Maslanka, AAMRL/TH, Wright-Patterson Air Force Base, OH 45433, and Flemming, C D NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431. Presented at the EMSA Annual Meeting, Milwaukee, WI, 7-12 August

Chlorotrifluoroethylene (CTFE) Oligomer: Effect on Livers of Fischer 344 Rats. 1988 Mattie, D R , M R Chase, AAMRL/TH, Wright-Patterson Air Force Base, OH 45433, Kinkead, E R , NSI Technology Services Corp , 101 Woodman Drive, Dayton, OH 45431, and Whitmire, R E , AAMRL/TH, Wright-Patterson Air Force Base, OH 45433 Presented at the EMSA Annual Meeting, Milwaukee, WI, 7-12 August

APPENDIX G

INVITED PRESENTATIONS

Conolly, R.B. 1987. Modeling of Chemical Carcinogenesis Presentation given at the University of Cincinnati Kettering Laboratories, Cincinnati, OH, 7 October.

Conolly, R.B. 1988. Computer Simulation of Chemical Carcinogenesis. Presentation given to the Department of Chemistry at Wright State University, Dayton, OH, 14 January

Conolly, R.B. 1988. Biological Data for Pharmacokinetic Modeling and Risk Assessment Presented at the EPA Workshop, Asheville, NC, 23-25 May

Conolly, R.B. 1988. Physiologically-Based Pharmacokinetic Modeling Presentation given to the Modeling Department of Pharmacology, Michigan State University, East Lansing, 19 July

APPENDIX H

1988 TOXIC HAZARDS RESEARCH UNIT GUEST SPEAKERS

DATE	TITLE	PRESENTER	THRU HOST
30 Nov 1987	The Immunoassay in Chemical Analyses	Dr. Bruce H. Hermann	Dr. R. Carpenter
07 Dec 1987	Simultaneous Kinetic Analysis of Free and Combined Chloride in Aqueous Solution	Dr Krishan Kuman	Dr. R. Carpenter
21 Dec 1987	Methanol/Water Reformation on Cu/ZnO Solids: Characterization of a Complex Heterogeneous System	Dr. Brian Goodby	Dr. R. Carpenter
08 Jan 1988	P450, Platelets, and the Puzzles of Pulmonary Pyrrolizidine Poisoning	Dr Robert A. Roth	Dr. A. Vinegar
20 Jan 1988	Physiologically-Based Pharmacokinetic Models for Inhaled Methanol: A Species Comparison	Dr. Vicki L. Horton	Dr. R. Conolly
25 Jan 1988	The Role of Topotherol in Adrenocortical Lipid Peroxidation and Steroidogenesis	Dr. Dee Ann Staats	Dr. R. Conolly
11 Jul 1988	Clearance Mechanisms in Lung and Extrapolation Modeling of Pulmonary Effects	Dr Gunter Oberdorster	Dr. A. Vinegar

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